DOCTOR OF PHILOSOPHY

Identification and characterization of genetic interactors of the Rho Guanine-nucleotide exchange factor Pebble in Drosophila

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Identification and characterization of genetic interactors of the Rho Guanine-nucleotide exchange factor Pebble in *Drosophila*

by

Margarethe Maria Draga

A thesis submitted for the degree of Doctor of Philosophy

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Abbreviations

Ala	Alanine
β-Gal	β-Galactosidase
bp	basepairs
C-terminal	Carboxy-terminal
cDNA	coding DNA
Chr	chromosome
Δ	delta, deleted
Df	deficiency, deletion
dH_2O	demineralized water
DAPI	4,6-diamino-2-phenylindole
EMS	Ethyl methane sulfunate
ERK	extracellular signal-related kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
Fig	Figure
GAP	GTPase activating proteins
GEF	guanine nucleotide exchange factor
GDI	Guanine nucleotide-dissociation inhibitors
GDP	guanosine diphosphate
glc	germline clone
GTP	guanosine triphosphate
GTPase	guanosine triphosphate (GTP)-binding proteins

HA	Hemagglutinin-epitope
HSPG	Heparan sulfate proteoglycan
hs	heatshock
kDa	kilo Dalton
LB-medium	Luria Bertani broth medium
m	milli 10^{-3}
µ	micro 10^{-6}
M	Molarity, mol/L
MAPK	mitogen activated protein kinase
min	minutes
MTOC	microtubule-organizing centre
N-terminal	amino-terminal
PCR	polymerase chain-reaction
PI3K	Phosphoinositide-3-kinase
PKA/PKC	protein kinase A /protein kinase C
ROCK	Rho kinase
RT	romm temperature
Ser	Serine
UAS	upstream activating sequence

Declaration

I declare that the following thesis is based on the results of investigations conducted by myself, and that this thesis is of my own composition. Work other than my own is clearly indicated in the text by reference to the relevant researchers or to their publications. This dissertation has not in whole, or in part, been previously submitted for a higher degree.

Margarethe M. Draga

I certify that Margarethe M. Draga has fulfilled the conditions of the relevant Ordinance and Regulations of the University of Dundee and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Dr. H.–Arno J. Müller

Summary

The gene *pebble* (*pbl*) encodes a Rho GEF required for the migration of mesoderm cells during *Drosophila* gastrulation. The spreading of mesoderm cells is controlled by the FGF signalling pathway acting through the FGF receptor Heartless (Htl). Pbl represents an important downstream component of this FGF pathway and activates the Rho GTPase Rac, but the regulation of Pbl by FGF signalling is unclear. Furthermore Pbl is required for the formation of the actin-myosin contractile ring during cytokinesis by activation of RhoA. The purpose of this work is to find molecular links between Pbl and the Htl signalling pathway and get insight into the localization and regulation of Pbl during mesoderm cell migration.

A genetic screen is carried out to find genes that interact with Pbl and are involved in mesoderm development. A gain-of-function variant of Pbl that causes defects in eye morphology was used to find genetic interactors. Results of a screen using chromosomal deletions and an EMS-based screen revealed candidates, which genetically interact with Pbl and are required for mesoderm cell migration.

In addition, a structure-function analysis of the Pbl protein was performed. The data revealed an important role of the PH domain for the localization of Pbl at the cell cortex. Moreover the PH domain is indispensable for the function of Pbl in mesoderm migration.

Furthermore an important role for the C-terminal tail of Pbl for the regulation of the protein was shown, which might be regulated by FGF signalling. The C-terminal tail is required for the stability of the protein outside the nucleus and it regulates the substrate preference of Pbl for Rac and Rho. Furthermore indication was found that the function of the C-terminal tail possibly is regulated by phosphorylation of Ser^{825} in the C-terminal tail. Mutation of this site affects the function of Pbl during mesoderm

migration but not in cytokinesis. Therefore phosphorylation of the C-terminal tail might regulate or enhance the exchange activity of Pbl for Rac.

The localization and function of Pbl depends on the PH domain and the C-terminal tail of Pbl. Both domains have distinct roles during Pbl function in mesoderm cell migration.

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1. Introduction

Cell migration is one of the most important processes during the development of multicellular organisms and is a major mechanism during immune responses and for the formation of tissues during wound healing. Cell migration requires to be stringently controlled through the interaction of several gene products to prevent misplacement of cells, which can result in various diseases like tumor progression and metastasis.

The reorganization of the actin cytoskeleton, the changes of cell shapes and the formation of cellular protrusions are major features for cells to become motile and to migrate. The small GTPases of the Rho family are key regulators of the actin cytoskeleton during morphogenetic movements. Rho GTPases are known to be required to control cellular processes like cell shape changes, adhesion and the cell cycle *(Hall, 1998)*. Guanine nucleotide exchange factors (GEFs) regulate the activity of Rho GTPases by catalyzing the exchange of bound GDP for GTP and hence promote activation of GTPases.

Drosophila melanogaster is an excellent model system for the analysis of cell migration. Most of the genes that are involved in the control of cell migration are conserved from flies to humans and therefore research data can readily be transferred to higher organisms. The Rho GEF Pebble (Pbl), a fly homologue of the mammalian proto-oncogene Epithelial cell transforming gene 2 (Ect2) is essential for mesoderm migration in *Drosophila (Schumacher et al., 2004; Smallhorn et al., 2004)*. The aim of this thesis is to elucidate the function of Pbl during cell migration in *Drosophila* to get a better understanding in cell migration in general.

1.1 Cell migration

Migration of cells is a major process during the development of an organism. Moreover cell migration is required for many processes in the adult organism and its mis-regulation is involved in many pathological conditions including cancer. Although cell migration is required for different processes like embryogenesis, wound healing, immune response, cancer and angiogenesis the mechanism underlying cell migration are very similar in different biological contexts. Migration is a cycling process, which includes polarization, formation of protrusions and adhesion and finally retraction of the cell *(Laufenburger and Horwitz, 1996; Ridley et al., 2003)*. In the following the mechanisms of these individual processes will be introduced.

Formation of cellular protrusions

Cell migration is triggered by signals, which either are chemokines, growth factors or extracellular Matrix (ECM) molecules. The cells respond to these cues with polarization and changes of the actin cytoskeleton. At the front the cells form cellular protrusions in the direction of migration, while protrusions retract at the rear edge of the cell. The protrusions can either be branched networks of filaments in lammelipodia or long, parallel actin filament bundles in filopodia *(Ridley et al., 2003)*. They differ between different cell types in organisms; primordial germ cells of zebrafish form bleb-like protrusions *(Blaser et al., 2006*) whereas leucocytes in mice form thick, actin-rich, pseudopodia-like protrusions *(Lammermann et al., 2008)*. In *Drosophila* mesoderm cells various types of protrusions form at the dorsal edge of the migrating cell *(Schumacher et al., 2004, Klingseisen et al., 2009)*.

Protrusions are formed by actin nucleation and actin polymerization. The major actin nucleating proteins are actin-related protein complex 2/3 (Arp2/3), Spire and Formins. The Arp2/3 complex is localized by Wave/Scar and Wiskott-Aldrich syndrome (WASP) family proteins at the plasma membrane and locally drives the

addition of actin subunits to the fast-growing barbed ends of the actin filaments *(Polard and Borisy, 2003)*. Arp2/3 binds to the tip of an existing actin filament and induces the development of a new actin filament branching out of the existing one. Arp2/3 is required for nucleation and branching of actin filaments, which grow rapidly and push the membrane forward *(Pollard and Borisy, 2003; Ridley, 2003; Goley and Welch 2006)*. Spire binds to the rear-facing pointed ends of filaments and prevents depolymerization (*Quinlan et al., 2005*). Formins induce actin nucleation of unbranched filaments. They are activated by Rho GTPases and associate with the growing barbed ends of actin filaments, where they stabilize the filament and promote nucleation *(Goode and Eck, 2007)*.

Many more actin-binding proteins are involved in actin polymerization in cellular protrusions. Among these capping proteins exist, which terminate the elongation of actin filaments. Furthermore there are proteins required for binding actin and other proteins cross-linking the actin cytoskeleton and linking it to the plasma membrane *(Saarikangas et al., 2010)*. The number of actin-binding, capping and cross-linking proteins is very high and therefore they are not introduced in detail. A few of them are going to be described later on, in processes relevant for this thesis.

Regulation of polarization and cell shape changes

Important signalling molecules that control cell shape changes and formation of protrusions during cell migration are the proteins Rac, Cdc42 and Rho, which belong to the Rho family of small guanosine triphosphate (GTP)-binding proteins (GTPases). In migrating fibroblasts Rac is activated at the front of the cell and regulates actin polymerization and the formation of lamellipodia and membrane ruffles (Fig. 1.1) *(Kraynov et al., 2000)*. Rac activates Arp2/3 complex via WAVE and induces actin polymerization and branching of actin filaments. On the other hand it inhibits actin filament disassembly by inactivation of ADF/Cofilin, which is an actin-depolymerizing

factor, through activation of Lim and Pak kinases *(Edwards et al., 1999; Isamil et al., 2009).*

Cdc42 is also active at the front of the cell by regulation of the formation of filopodia and directed migration *(Nobes and Hall., 1995).* Cdc42 is required for the polymerization and bundling of F-actin during extension of filopodia. It activates the Arp2/3 complex through WASP proteins and inhibits actin filament disassembly by activating PAK kinases *(Hall, 1998; Edwards et al., 1999*). Furthermore Cdc42 is required for the formation of an actin network and the establishment of polarity of the cell. Cdc42 localizes the Par/PKC complex to the front of the cell. In addition it regulates the localization of the microtubule-organizing centre (MTOC) and Golgi in front of the nucleus, so that both can regulate the vesicle transport towards the leading edge (Fig. 1.1) (*Etienne-Manneville and Hall, 2001; Gotlieb et al., 1981; Gundersen and Bulinski, 1988; Kupfer et al., 1983; Kupfer et al., 1982*). This way Cdc42 is required for microtubule-regulated transport of proteins required for formation of protrusions to the leading edge *(Ridley, 2003).*

Rho is required for the bundling of F-actin and MyosinII to contractile actin-myosin bundles like stress fibers and it regulates the formation of new focal adhesion sites (Fig. 1.1) *(Hall, 1998; Pelham and Chang, 2002; Ridley and Hall, 1994).* Rho induces actin polymerization at focal adhesions by activation of the Formin Diaphanous (Dia) and the Rho kinase (ROCK), which in turn activates Myosin light-chain kinase (MLC). MLC regulates the activity of MyosinII, an actin-dependent motor protein, which drives the contractility of the cell (*Totsukawa, 2000; Riento and Ridley, 2003*).

The actin polymerization and elongation at the front and the actin-myosin filament contraction at the rear end are the driving force of cell migration, with Rac and Rho antagonize each other along the way. Rac is active at the front and suppresses

activation of Rho, whereas Rho is active at the sides and the rear of the cell suppressing Rac function in protrusion formation *(Worthylake and Burridge, 2003; Xu et al., 2003).*

Formation of cell adhesion

The cells form adhesive contacts to neighbouring cells and the ECM to move forward (Fig. 1.1). The protrusions attach to the surrounding matrix to stabilize; furthermore adhesions transfer the propulsive force of the cytoskeleton to a substrate to move forward. Migration promoting receptors, called Integrins support adhesion of migrating cells to the ECM or neighbouring cells and link actin filaments to signalling adaptors, like for instance Paxillin. After activation they form Integrin clusters and activate signalling pathways through GTPases and Phospholipids that are required for strengthening of adhesion sites, organization of the actin cytoskeleton and generation of cell polarity *(Geiger et al., 2001).* The formation of Integrin clusters, so called focal adhesions is mediated through Focal adhesion kinase (FAK) and Rho and involves the assembly of actin stress fibres by MyosinII.

However the attachment of the migrating cell to the ECM has to be released, at the leading edge to form new cellular protrusions and at the rear to promote retraction *(Webb et al., 2002).* FAK forms and disassembles focal adhesion *sites (Ridley, 2003; Tomar and Schlaepfer, 2009),* together with Src kinase and Rac FAK is required for the so called adhesion turnover, the disassembling of the adhesion sites, which goes along with MyosinII contractility. Adhesion turnover and contraction of actin-myosin network are required for the retraction of the rear edge and the forward movement of the cell *（Henson et al., 1999; Medeiros et al., 2006; Vallotton et al., 2004; Verkhovsky et al., 1999; Zhang et al., 2003）.*

A general model for cell migration taken together all previously described mechanisms is drawn below:

Cells migrate towards guidance cues. These signals activate receptors, which induce different signalling cascades including activation of tyrosine kinases, lipid kinases and Rho GTPases. All these signals lead to polarization of the cell, formation of cellular protrusions and the formation of adhesions. The disassembling of adhesions and retraction at the rear completes the migration cycle and it starts again with the formation of new protrusions and adhesions (Fig. 1.1) *(Ridley et al., 2003).*

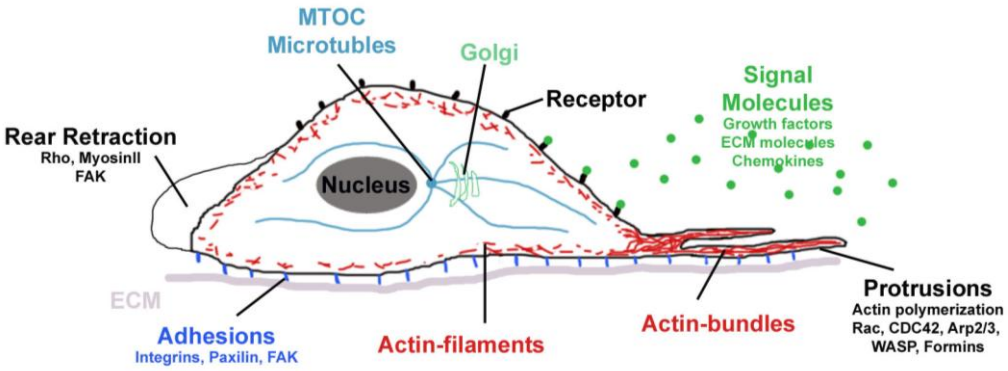


Fig. 1.1 The migrating cell
After binding of signalling molecules to receptors, Rho GTPases are activated. Cdc42 regulates the polarity of the cell and the formation of filopodia. Rac induces the formation of lamellipodia and regulates the adhesion turnover. Rho is required for the retraction of the rear end of the cell through the formation of actin stress fibres and MyosinII. The cell forms protrusions towards the direction of the signalling molecules and adhesions to the ECM and neighbouring cells. The cell migrates through the force of retraction and adhesion disassembling (modified after *Petit et al., 2002; Ridley et al., 2003*).

The key regulators of cell migration, like Phospholipids, growth factors and Rho GTPases are going to be explained more detailed in the next paragraphs.

1.2 Regulation of the actin cytoskeleton by small GTPases of the Rho family

Rho GTPases belong to the Ras superfamily of small GTPases (~21 kDa) and there are 22 members of this family known in human *(Rossman et al., 2005)*, eight in *Drosophila*, five in *C. elegans* and 15 in *Dictyostelium (Raftopoulos and Hall, 2004)*. The first Rho GTPases that were identified and since have been investigated most thoroughly are RhoA, Rac and Cdc42 (*Ridley and Hall, 1992; Hall, 1994; Macheski and Hall, 1996; Ridley, 1995*).

In the last paragraph the role of Rho GTPases in cell adhesion and cell migration was described. Furthermore Rho GTPases regulate many other cellular processes like morphogenesis, axon guidance, polarization, phagocytosis, cytokinesis, cell growth and cell survival *(Schmidt and Hall, 2002, Rossman et al., 2005)*. Rho GTPases are highly regulated and switch between an inactive GDP bound state and an active GTP bound state *(van Aelst and D'Souza-Schorey, 1997)*. The cycling between the active and the inactive state is regulated by Guanine nucleotide exchange factors (GEF), GTPase activating proteins (GAP) and Guanine nucleotide-dissociation inhibitors (GDI). GEFs catalyze the GDP release and exchange for GTP and thus activate the Rho protein. GAPs enhance the very low intrinsic GTPase activity of Rho proteins and suppress the Rho activity. Finally GDIs keep the Rho GTPases in a GDP bound state in the cytosol *(Rossman et al., 2005).*

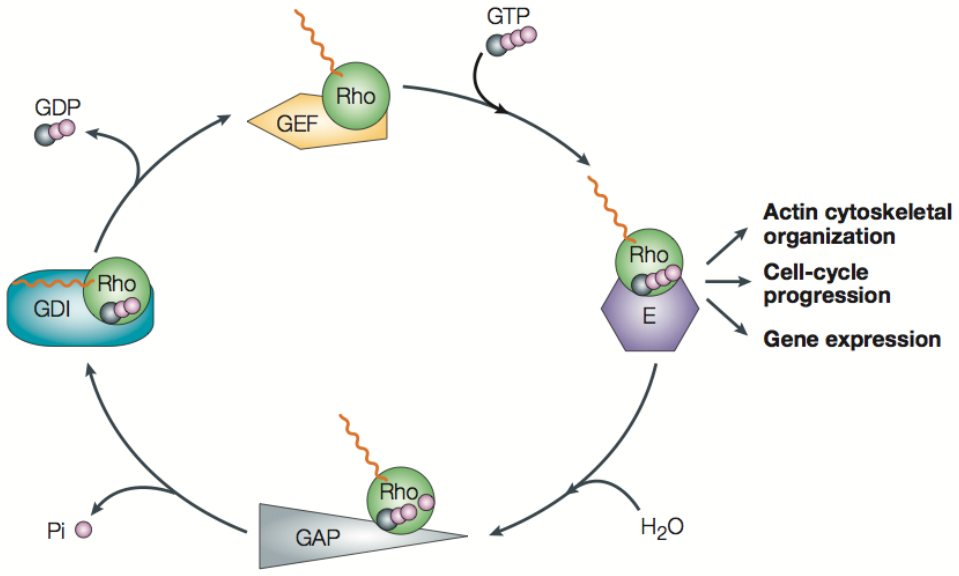


Fig. 1.2 Regulation of Rho GTPases.
Rho GTPases cycle between an active, GTP bound and an inactive, GDP bound state. GEFs catalize the exchange of GDP for GTP and activate the Rho GTPase, which can bind and regulate effectors (E). GAPs inactivate Rho GTPases by hydrolysis of GTP. GDIs keep GDP bound Rho GTPases in the cytosol (*Rossman et al., 2005*).

Rho GEFs

Rho GEFs activate Rho GTPases by interaction of their DH domain with the switch region of the GTPase; there are over 70 Rho GEFs in humans and the DH (Dbl homology) domains are named after the first identified mammalian Rho GEF Dbl (*Eva et al., 1988; Hart et al., 1991*). DH domains are required for catalyzing the exchange of GDP for GTP in the Rho GTPase. The structure similarities of DH domains are very low allowing a very specific interaction of Rho GEFs with their Rho GTPases. Exchanges of single amino-acids in the switch region of the Rho GTPase or the DH domain of the GEF result in GEFs not being able to bind the Rho GTPases. For instance a mutation in the DH domain of ITSN-L, normally a GEF for RhoA, results in ITSN-L not being able to bind and activate RhoA. Moreover a mutation in Dbs can induce binding to Rac1, which is usually not a substrate for Dbs (*Karnoub, A. E. et al. 2001; Cheng, L. et al. 2002; Snyder, J. T. et al. 2002*).

Many GEFs contain Pleckstrin homology (PH) domains located C-terminally to the DH domain. PH domains are found in many signalling molecules that bind to phospholipids. In some GEFs PH domains function as membrane-anchor and additionally support the exchange activity of the DH domain for the Rho GTPase (*Ferguson et al., 1995; Liu, X. et a., 1998; Rossman and Campbel, 2000*). However not all Rho GEFs posses PH domains, in some GEFs the PH domains are replaced by BAR (Bin, Amphihysin, Rvs) domains, which have a different structure than PH domains, but can bind to phospholipids as well (*Takei et al., 1999*).

Additionally there is another GEF protein family, which does not contain DH-PH domains. There are 11 DOCK family member proteins that are related to DOCK1 and contain DOCK homology regions 1 and 2 (DHR1, DHR2) (*Brugnera, E. et al. 2002; Meller et al., 2002; Namekata et al., 2004*). The DHR2 domains (also called CZH domain or Docker domain) show catalytic activity and promote nucleotide exchange in Rho GTPases. Furthermore they contain a second conserved region DHR1/CZH1 that can bind to phospholipids and is important for the positioning and promoting of Rho GTPase activity (*Côté and Vuori, 2007*). The DOCK protein family is divided into four subfamilies, which differ in their regulatory domains and specificities for Rac and Cdc42 (*Meller et al., 2005 ; Côté and Vuori, 2007*). DOCK A and B proteins show exchange activity for Rac, whereas Dock D proteins can activate Cdc42. Proteins of the Dock C subfamily show exchange activity for both Rac and Cdc42 (*Brugnera et al., 2002*; *Côté and Vuori, 2002; Miyamoto et al., 2007).*

In the activated state Rho GTPases control the activation and regulation of many downstream targets that regulate actin dynamics *(Raftopoulos and Hall, 2004).* Upstream of Rho GTPases many processes are regulated through receptor tyrosine kinases (RTKs) *(Schiller 2006)* and additionally by phosphoinositides.

Regulation of Rho GTPases by Phosphoinositides

Phosphoinositides are essential regulators of many cellular processes. They are involved in many human diseases and function as signalling lipids during inflammation, cancer and metabolic diseases *(Saarikangas et al., 2010)*.

Phosphoinositide-3-kinase (PI3K) is activated by a number of growth-factor-receptors and catalyzes the phosphorylation of the phospholipid phosphatidylinositol (4,5)-bisphosphat (PtdIns(4,5)-P_2) to phosphatidylinositol (3,4,5)-triphosphat (PtdIns(3,4,5)-P_3) *(Cantley et al., 1991)*. The phosphatase PTEN hydrolyses PtdIns(3,4,5)-P_3 into PtdIns(4,5)-P_2.

Phosphoinositides regulate actin cytoskeleton dynamics mainly through Rho GTPases. Phosphoinositides can directly bind the PH domains of GEFs for Rho GTPases, recruit them to the plasma membrane and induce their activation. For instance Vav2 and Sos1, which are GEFs for Rac1, are activated by binding of PI(3,45,)P3 to their PH domains *(Das et al., 2000)*. Additionally phosphoinositides regulate Arf (ADP ribosylation factor) GTPases during membrane traffic. Arfs regulate the production of PI(4,5)P2 at the Golgi and at the plasma membrane. Furthermore Arf6 was shown to be involved in the recruitment of Rac to the plasma membrane (*D'Souza-Schorey and Chavrier, 2006; Myers and Casanova, 2008).*

Besides the interaction with Rho GEFs, PI(3,4,5)P3 binds to a large number of proteins containing lipid binding domains like PH, PTB, PX and FYVE domains and recruits them to the cell membrane. Furthermore PI(3,4,5)P3 binds to the serine/threonine protein kinase B (PKB), also called Akt. Akt/PKB is downstream of growth factor regulated signalling pathways and required for several cellular processes *(Liao and Hung, 2010)*. PI(4,5)P2 binds to actin-binding proteins that induce actin filament assembly and regulates their activity *(Saarikangas et al., 2010)*.

The localization of Phosphoinositides depends on the localization or local activation of PI3K and PTEN, which regulate the amount of PI(3,4,5)P3 and PI(4,5)P2. PI3K generally localizes to the parts of the cell where an actin-network is formed, whereas PTEN localizes to the edges where contractile actin-myosin structures are established *(Saarikangas et al., 2010)*. For instance during cell migration in *Dictyostelium* PI3K and its product PI(3,4,5)P3 are localized to the leading edge of migrating cells during chemotaxis, where cellular protrusions develop. PTEN is at the retracting tail, suppressing the formation of lamellipodia *(Van Haastert and Veltman, 2007; Sasaki and Firtel, 2006; Wessels et al., 2007)*.

1.3 Regulation of cell migration by growth factors

Growth factors regulate a huge variety of processes that require cell specification and cell movements during development. Many Rho GTPases and Phosphoinositides are regulated by growth factor signalling.

Different types of growth factors like vascular endothelial growth factors (VEGF), epidermal growth factors (EGF), fibroblast growth factors (FGF), platelet-derived growth factors (PDGF) and Ephrins activate RTK and have distinct functions during development of an organism. The most common signalling pathway activated by growth factors is the mitogen activated protein kinase (MAPK) pathway. Besides MAPK activation different growth factors commonly induce a panel of signal transduction pathways through PI3K/Akt, phospholipase Cγ/protein kinase C (PLCγ/PKC) and GTPases *(Schiller, 2006)*.

FGFs and their receptors are required for cell specification and cell migration during gastrulation in many organisms, including *Drosophila*. The FGF family in human contains 22 FGF ligands and 4 receptors (*Ornitz and Itoh, 2001*). Whereas three FGF ligands and two receptors exist in *Drosophila* (*Gabay et al., 1997; Wilson et al.,*

2000). However the protein structure and functions of FGFs and their receptors are very good conserved between species.

1.3.1 FGF signal transduction

The most common pathway activated by FGFs is the MAPK cascade (Fig. 1.3). FGFR are activated by the binding of FGFs and heparan sulphate proteoglycans (HSPGs). FGF ligands, FGFR and the HSPGs form a complex, which leads to dimerization and autophosphorylation of the receptor *(Schlessinger, 2000; Böttcher and Niehrs, 2005)*. Compared to many other RTKs FGF receptors lack a Grb2 binding site and therefore need to bind to an intermediate docking molecule to activate MAPK (*Michelson et al., 1998*; *Imam et al., 1999; Vincent et al., 1998)*. In vertebrates, FGF receptor substrate 2 (FRS2) functions as such a signalling mediator that links the adaptor protein growth factor receptor-bound-2 (Grb-2) to the FGF receptor *(Kouhara et al., 1997)* and binds to the protein tyrosin phosphatase Shp-2. Grb-2 binds via its SH2 and SH3 domains to FRS2 and to Son of sevenless (Sos). Sos is a GEF and activates Ras1 GTPase. Ras1 activates the Serine/threonine kinase Raf, which then activates Ser/Thr kinase MEK. MEK phosphorylates and activates MAPK. The activated form ERK is finally translocated into the nucleus where it activates and regulates transcription factors, other kinases and cytoskeletal proteins by phosphorylation.

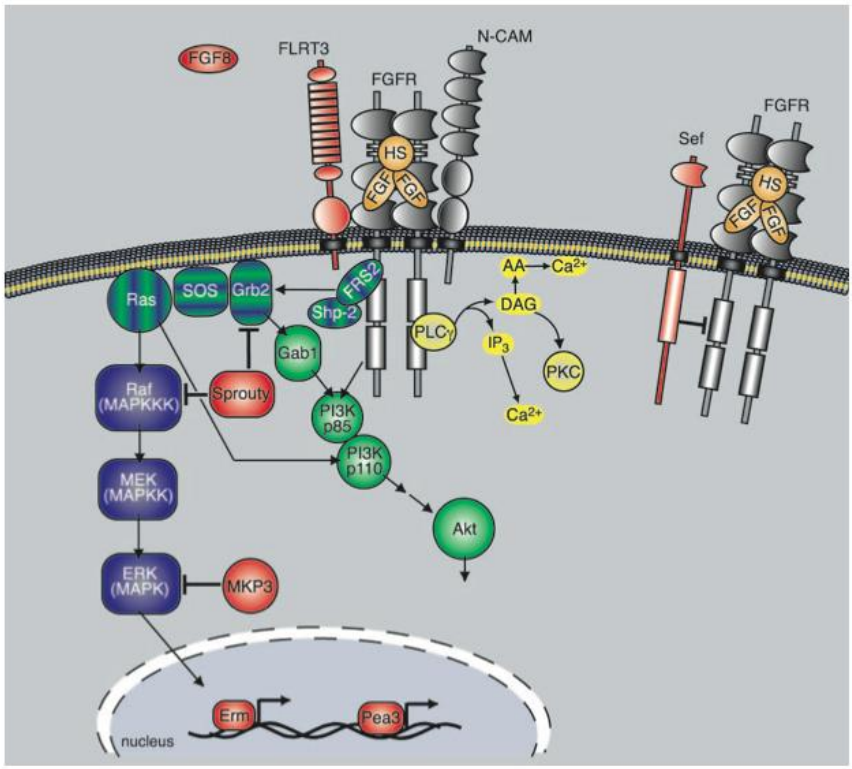


Fig. 1.3 Intracellular signalling pathways activated through FGFs
Activation of the FGFR by FGFs and HSPG leads to autophosphorylation of the receptor. The adaptor Grb2 binds via the signalling mediator FRS2 to the receptor and activates the Ras/MAPK and PI3K/Akt pathways. PI3K/Akt can be activated through direct binding of PI3Kp85 to the receptor and through Ras GTPase activation as well. Sprouty can antagonize MAPK activation via inhibition of either Grb2 or Raf1. Activation of the PKC pathway happens via direct binding and phosphorylation of PLC by the receptor (*Böttcher and Niehrs, 2005*).

Furthermore FGF signalling can induce activation of PI3K/Akt signalling in three different ways (Fig. 1.3). After recruitment of Grb-2 by FRS2 to the receptor, Grb-2 either binds to Gab1 or to Ras1 and induces the activation of PI3K/Akt pathway parallel to MAPK activation. Additionally the FGFR itself can bind to PI3Kp85 directly and induce the activation of PI3K/Akt *(Böttcher and Niehrs, 2005).*

Phospholipase C (PLCγ) /Ca2+ pathway is activated by FGF signalling as well (Fig. 1.3). PLCγ binds directly to a phosphorylated tyrosine of the FGFR. Then it hydrolyzes PI(4,5)P2 to form inositol-1,4,5-triphosphat (IP3) and diacylglycerol (DAG). IP2 induces the release of Ca^{2+} and DAG activates protein kinase C (PKC). PKC is required for many different cellular processes including cell polarization and migration.

Negative regulators of MAPK activation are the Sprouty (Spry) proteins. The first Spry protein was found in *Drosophila* as an antagonist of FGF induced tracheal cell migration. In Spry mutants FGF signalling occurs in an unrestricted fashion and results in formation of ectopic tracheal branches *(Hacohen et al., 1998)*. Later on it was shown that Sprouty is a general regulator of RTK activated MAPK cascade *(Kim and Bar-Sagi, 2004; Cabrita and Christofori, 2008)*. In both vertebrates and *Drosophila* Spry is antagonizing growth factor activated MAPK signalling. There are four Spry proteins in vertebrates and only one in *Drosophila*. Sprys are activated by phosphorylation, bind to Grb2 and inhibit the binding of Grb2 to either RTK or to the adaptor protein Shp2 *(Hanafusa et al., 2002)*. Other reports show that Sprys act downstream of Ras binding to Raf and inhibiting MAPK activation there *(Sasaki et al., 2003)*. Additionally there are Sprouty-related proteins, called Spreds, which have the same structure and negative regulatory function on MAPK activation like Sprys. Spred and Sprys seem to have distinct and specific roles in controlling RTK regulated MAPK activation *(King et al., 2005; Cabrita and Christofori, 2008).*

1.3.2 FGF signalling during embryonic development

FGF signalling is required for many processes during embryonic development. For instance FGFs regulate body axis formation in early embryogenesis of Xenopus, mouse and zebrafish *(Amaya et al., 1991; Deng et al., 1994; Yamaguchi et al., 1994; Griffin et al., 1995).* Later in development FGFs regulate the specification and maintenance of the mesoderm and morphogenetic movements during gastrulation.

The mesoderm development in the chicken embryo is going to be explained as an example for FGF regulated mesoderm cell migration in vertebrates. Another example for FGF regulated cell migration is given for tracheal tube formation in later embryonic development in the *Drosophila* embryo.

Mesoderm cell migration in the chicken embryo

The early chicken embryo consists of two cell layers, called epiblast and hypoblast. Cells from the epiblast migrate away from the primitive streak and ingress. Then the cells migrate between the epiblast and the hypoblast to form the mesoderm *(Lawson and Schoenwolf, 2001).* The migration of the cells is regulated by two FGF ligands, FGF8 and FGF4, and the FGFR1 receptor. Both ligands are expressed in the streak, with partially overlapping expression patterns. However the expression patterns change during development. FGF8 is initially expressed in the anterior streak and the node, but later on it is excluded from the same parts. FGF4 is expressed in all cells of the streak. FGF8 is highly expressed at the position where the cells migrate out of the streak, suggesting that FGF8 acts as repellent and drives the cells away. The cells migrate now towards the FGF4 signal, which functions as an attractant. The direction of the migration depends on the position of the cells and on how much of the repelling FGF8 signal and the attracting FGF4 signal they receive (Fig. 1.4) *(Yang et al., 2002).*

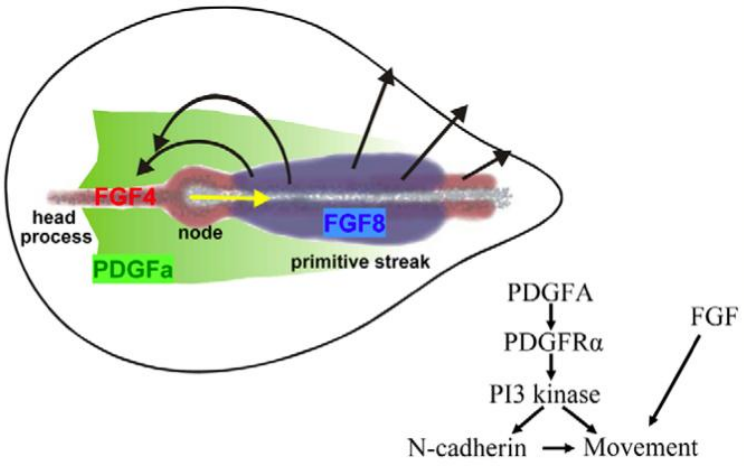


Fig. 1.4 Migration of the mesoderm cells in the chicken embryo is regulated by FGF and PGDF signalling
Expression pattern of FGF8 (blue) and FGF4 (red); in the primitive streak the expression of FGF8 and FGF4 overlaps (indicated by the purple color). The black arrows show the direction of the cell movement. FGF8 acts as chemo-repellant, so the cells move away from the primitive streak. The FGF4 signal in the anterior part of the streak attracts the cells towards the head process and the notochord. PDGF is expressed on both sides of the primitive streak (green) and controls together with FGF signalling the migration of the cells *(Yang et al., 2008).*

When they reach their final position the cells are specified to become precursor cells for mesodermal structures. Cells in the anterior streak become somites, whereas posterior cells become extra-embryonic mesoderm and haematopoietic stem cells. Cells in the middle of the streak give rise to intermediate and lateral plate mesoderm *(Limura et al., 2007; Psychoyos and Stern, 1996).*

Furthermore it was shown, that PDGF signalling and the activation of PI3K/Akt pathway are also required for mesoderm cell migration. PDGFA and the receptor PDGFRα are required for the migration of the cells away from the primitive streak, by regulating the expression of N-Cadherin and the activation of the PI3K/Akt pathway *(Yang et al., 2008).* The example of the development of the chicken embryo shows that two different growth factors may have distinct roles during mesoderm cell migration.

FGF regulation of Tracheal morphogenesis in Drosophila

Two FGF receptors exist in *Drosophila*. Heartless (Htl) is required for cell specification and migration during mesoderm development *(Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998; Shishido et al., 1993; Shishido et al., 1997)* and Breathless (Btl) regulates cell migration during tracheal morphogenesis *(Klämbt et al., 1992; Sutherland et al., 1996).*

The tracheal system in *Drosophila* develops from segmentally repeated clusters of ~80 epidermal precursor cells. The cells are assembled by cell migration and elongation into epithelial tubes that transport oxygen from the environment to all tissues *(Manning and Krasnow, 1993; Samakovlis et al., 1996).* The FGF receptor Breathless (Btl) and its ligand Branchless (Bnl) are required for the regulation of the primary branching. Bnl surrounds the tracheal sacs and induces migration of the cells of the first six tracheal branches and provides guidance cues (Fig. 1.5).

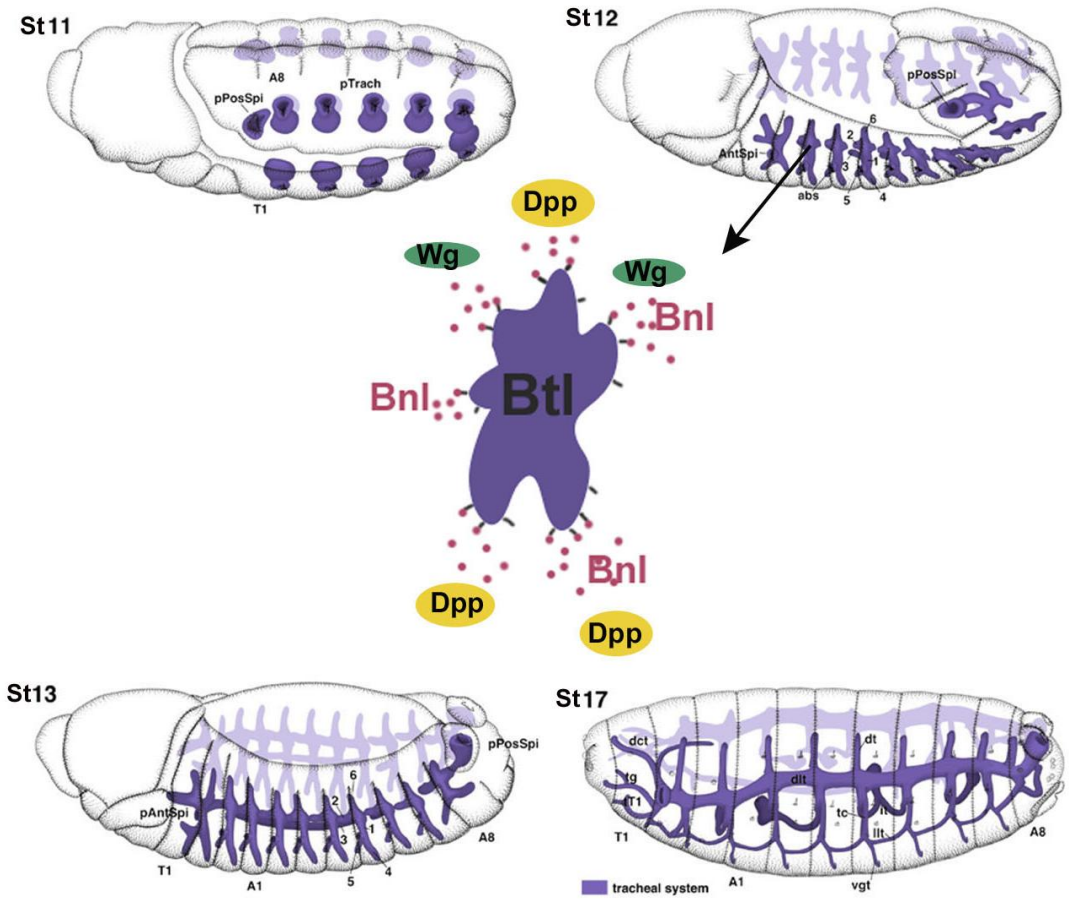


Fig. 1.5 Tracheal morphogenesis.
In stage (st) 11 of embryogenesis, sacs of epidermal cells are arranged in every segment (purple). Stage 12: The cells of the primary branches express Btl receptor and migrate out of the sacs towards the Bnl ligand expression. Wg signalling is required for the specification of the dorsal trunk (dt), Dpp for the formation of the dorsal branches and the lateral trunk (dlt). The cells form tracheal tubes, while migrating. Stage 13: new tubes branch out of the primary tubes and the cells elongate and finally form a network of tracheal tubes that transport oxygen to every tissue in the body (stage 17, end of embryogenesis) (modified after *Hartenstein, 1993; Petit et al., 2002*).

The cells migrate out of the cell clusters with the tip cells (the cells at the front) forming filopodia and lamellipodia towards the Bnl signal. During the migration the cells fuse with each other and form tubes *(Klämbt et al., 1992; Sutherland et al., 1996; Affolter and Caussinus, 2008).* Then secondary branches sprout out of the primary branches; this is controlled by the transcription factor Pointed (Pnt). Under the control of serum response factor (SRF) *pruned* the secondary branches branch out into many terminal branches, which represent long cytoplasmic extensions that transport oxygen directly into the tissues *(Guillemin et al., 1996).*

The regulation of the patterning of the tracheal cells depends on Wg/Wnt, Decapentaplegic (Dpp) and Hedgehog (Hh) signalling. Wg is required for the specification of the dorsal trunk cells, while Dpp is required for the formation of the dorsal branches and the lateral trunk and Hh controls the formation of the tracheal branches. In embryos mutant for these genes and their downstream targets the tracheal cells do not migrate. However in mutants of the FGFR Btl and FGF Bnl the cells do not migrate either, therefore the activation of the FGF signalling pathway is indispensable *(Klämbt et al., 1992; Sutherland et al., 1996).* Upon binding of the ligand Bnl to the receptor Btl and the HSPGs the receptor is activated. Sugarless and Sulfateless are two enzymes, which act in HSPGs synthesis and are required for the activation of the receptor and for proper activation of MAPK kinase via Btl and Htl *(Lin et al., 1999).* *Sugarless* and *Sulfateless* mutants show defects in tracheal morphogenesis and during mesoderm migration, presumably both are generally required for signal transduction through FGF receptors *(Lin et al., 1999).*

Autophosphorylation of the receptor leads to activation of the adaptor protein Downstream-of-FGF (Dof, also Heartbroken or Stumps), which is indispensable for activation of Ras/MAPK *(Vincent et al., 1998; Imam et al., 1999; Lin et al., 1999).*

1.4 The role of EMT in cell migration

Cell migration occurs in different tissues and during different stages of development and in the adult organism. While cells like leucocytes and hemocytes, which are in circulation, start to migrate upon activation other cells have to break out of epithelial cell sheets to migrate and undergo Epithelial to Mesenchymal Transition (EMT). EMT is a process where the epithelial cells undergo dramatic changes of their cytoskeleton like change their polarity, loose their adherens junctions that exist between neighboring cells to form a tight epithelial cell layer to transform from epithelial cells to

become mesenchymal cells (Fig. 1.6) *(Thiery and Sleeman, 2006; Acloque et al., 2009).* This process can also be reversed and is then called Mesenchymal to Epithelial Transition (MET).

The first process in the developing embryo where EMT occurs is during gastrulation, when the three germ layers mesoderm, ectoderm and endoderm develop. At the beginning of gastrulation in multicellular organisms the formation of the mesoderm out of the primitive epithelium involves EMT. In the chicken embryo a group of cells migrates from the epiblast to the midline to generate the primitive streak. The cells undergo EMT and internalize to form mesoderm and endoderm, the cells remaining in the epiblast become ectoderm *(Acloque et al., 2009).* Also during mouse gastrulation the mesodermal precursor cells undergo EMT and migrate away from the primitive streak *(Ciruna and Rossant, 2001).* In *Drosophila* the embryo exists as a blastoderm epithelium. The ventral epithelial cells invaginate into the embryo, undergo EMT and migrate to form the mesoderm *(Leptin and Grunewald 1990; Bate et al., 1985).*

EMT is also required for other developmental processes during embryogenesis, for example, neural crest cell migration in vertebrates *(Duband et al., 1995)* or tracheal cell migration in *Drosophila (Ghabrial et al., 2003).*

EMT is induced by transcription factors, the major one is Snail, that are activated by a number of signalling events (Fig. 1.6). Wnt signalling is required for mesoderm formation involving EMT in different organisms *(Kelly et al., 1994; Smith and Harland, 1991; Sokol et al., 1991).* Additionally the transforming growth factor-β (TGF-β) and the TGFβ sub-family members Nodal, Vg1 and BMP are major regulators of EMT *(Shah et al., 1997; Kimelman, 2006; Raible, 2006).* Furthermore Notch and FGF signalling regulate EMT during different processes *(Ciruna and Rossant, 2001; Timmerman, 2004; Cornell and Eisen, 2005).* Signalling events regulated by Wnt, FGF

and TGF-beta subfamily members induce EMT. These signals regulate the expression of the transcriptionfactors Snail and Twist. Snail is required for the down-regulation of epithelial genes whereas Twist activates the expression of mesenchymal genes. The down-regulation of epithelial genes like Crumbs and E-Cadherin results in a loss of adherens junctions and cell polarity. Furthermore Snail is required for the break down of the basal membrane, which allows the former epithelial cell to migrate out of the cell layer and become mesenchymal (fig. 1.6.).

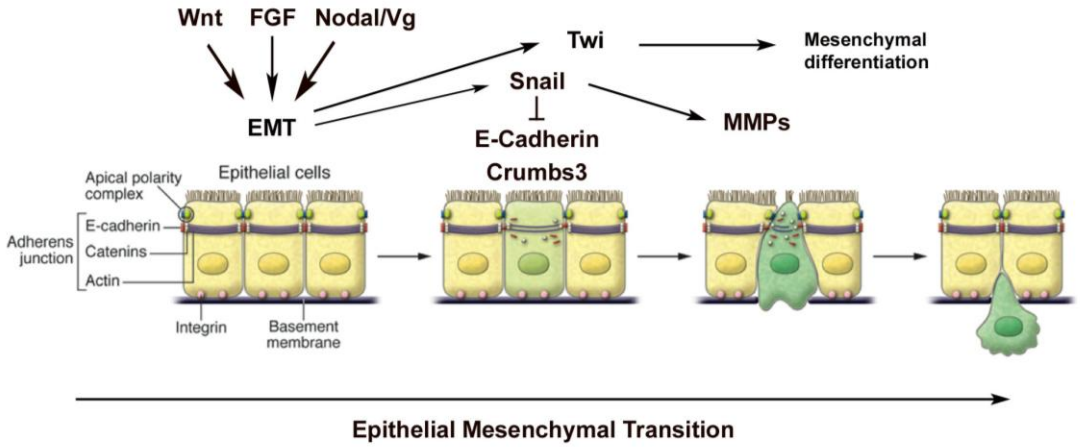


Fig. 1.6 Epithelial Mesenchymal Transition.
EMT is induced in epithelial cells by the Wnt, the FGF and TGF-β signalling pathways. The transcription of the cells is changed by Snail and Twi. Epithelial genes encoding for adherens junctions and polarity proteins are down-regulated. The expression of mesenchymal genes, encoding for proteins required for differentiation and migration are up-regulated. After the adherens junctions disassemble and the cells loose their epithelial characteristics and become mesenchymal, finally basement membrane breakdown is induced by Snail and the cells migrate out of the epithelial cell layer (modified after *Acloque et al., 2009*).

zinc-finger transcription factors like Snail and Slug are activated and they down-regulate the expression of epithelial genes. These genes encode for proteins required for maintenance of cell polarity and cell adhesions. For instance the expression of E-Cadherin, which is a major component of cell adhesions, is down-regulated by Snail *(Batlle et al., 2000; Cano et al., 2000; Haijra et al., 2002)*. Furthermore Snail represses the transcription of polarity proteins including Crumbs3 and Lgl2 *(Aigner et al., 2007; Spaderna et al., 2008)*, resulting in the Par6/PKC complex and tight junction proteins not being localized properly. Snail is the modulator of EMT in many organisms. During mesoderm development in *Drosophila* Snail suppresses DE-Cadherin transcription,

which results in the dissolution of cell adhesions. The transcription factor Twist initiates the transcription of genes required for mesoderm development *(Leptin, 1991; Oda et al., 1998).* Snail is expressed to induce mesoderm formation in mice gastrulation *(Carver et al., 2001).* Additionally Snail and Slug play essential roles during the neural crest cell migration in Xenopus and chicken embryos *(Nieto, 2002).*

After changes of transcription and the loss of polarity and adherens junctions, the cells need to pass through the basal lamina and finally away from the epithelial cell layer. This happens by activation of metalloproteases (MMP) and RhoA. MMP2, MMP3 and MMP9 are activated by Snail and disintegrate the basal membrane *(Jorda, M., et al. 2005; Miyoshi, A., et al. 2004).* The loss of RhoA at the basal membrane of the cells leads to basement membrane breakdown in the chicken primitive streak *(Nakaya et al., 2008).*

EMT is not only required for cell migration during embryonic development and in the adult organism, but also a major process during tumor progression in a majority of human cancers. Most tumors derive from epithelial cell layers, undergo EMT and invade other tissues *(Thiery, 2002).* However the identification of tumor cells undergoing EMT seems very difficult and even the fact that some tumors show expression of E-Cadherin means that the downregulation of E-Cadherin is not enough to explain EMT *(Tarine et al., 2005; Thompson et al., 2005).*

1.5 Development of Drosophila melanogaster

Drosophila melanogaster, the fruit fly, is a well-described model organism for more than 100 years. The genome is sequenced and contains approximately 13.600 genes. Most of the genes, required for embryonic development are evolutionarily conserved. Therefore *Drosophila* provides a very good system to study processes like

signalling transduction with relevance to human disorders. In this study it is used to investigate aspects of the FGF signalling pathway during mesoderm cell migration.

1.5.1 Early development of the *Drosophila* embryo

In the *Drosophila* embryo the mesoderm evolves out of the blastoderm epithelium, which is the first epithelium and develops during cellularization. After fertilization the embryo undergoes a superficial cleavage, which is characteristic for all insects and produces a syncytial embryo. Meaning the zygote undergoes nine simultaneous nuclei divisions without cytokinesis. Then the nuclei migrate towards the periphery of the embryo and undergo four additional divisions. At the interphase of cycle 14 furrow canals are formed out of the plasma membrane and by insertion of intracellular membrane transport and develop slowly towards the middle of the embryo. This so called slow phase is followed by a fast phase after which the furrow canals surround each nucleus and fuse with each other. Cell adhesions are formed between the cells, which are now polarized and the embryo exists as a cellular blastoderm epithelium that surrounds the yolk *(Müller and Bossinger, 2002)*. In *Drosophila* the mesoderm develops from the most ventral cells of the blastoderm epithelium.

1.5.2 The Specification of the mesoderm

The determination of the dorsal-ventral pattern of the embryo depends on the maternal components of several genes, including the transcription factor *dorsal*. A gradient of the maternal Dorsal protein is established within the dorsal-ventral axis of the syncytial blastoderm. In the ventral cells the Dorsal protein is transported into the nucleus while it stays in the cytoplasm of the dorsal cells *(Roth and Nusslein-Volhard, 1989).* Dorsal activates the expression of zygotic genes *twist (twi)* and *snail (sna)* in the ventral cells. Both are required for mesodermal cell fate: Twi activates mesodermal genes and Sna represses ectodermal genes and is required for EMT (*Leptin, 1991*). The

important role of Twi and Sna during mesoderm development becomes apparent in mutants of these genes, where the ventral cells fail to invaginate and the mesoderm is not formed.

At the beginning of gastrulation the most ventral epithelial cells expressing Twi, invaginate towards the middle of the embryo. Invagination is regulated through the transcription factor Twi (Fig. 1.7). Twi activates the ligand Folded Gastrulation (Fog), which binds to a hypothetic Fog receptor and activates a pathway involving Concertina and RhoGEF2. RhoGEF2 activating RhoA and Rho Kinase (ROCK) apically, controls the formation and contraction of an actin-myosin network (Fig. 1.7) (*Dawes Hoang et al.,2005*). This actin-myosin network leads to apical surface constriction of the mesoderm cells resulting in their invagination (*Dawes Hoang et al., 2005; Barrett et al., 1997*).

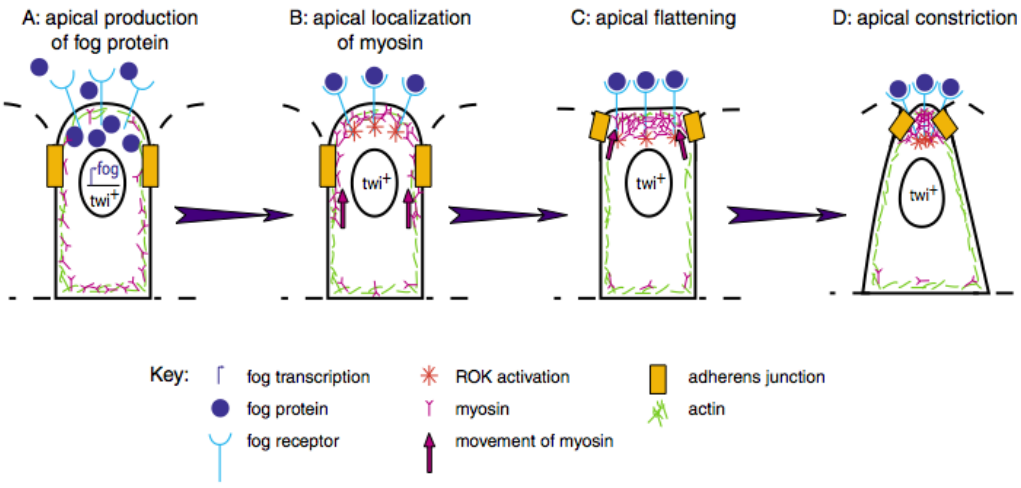


Fig. 1.7 Model of apical surface constriction.
(A) Twi expression specifies mesodermal cell fate in the ventral cells and activates the expression of the ligand Fog. (B) Fog binds to a hypothetic receptor on the apical side and activates RhoA and ROCK via Concertina and RhoGEF2. (C) RhoA and ROCK regulate the contraction of myosin and actin, which results in a flattening of the apical cell surface and finally creating a force that leads to apical constition of the cell (D) (*Dawes Hoang et al., 2005*).

After invagination the mesodermal cells form an epithelial tube, which disassembles in the interior of the embryo (Fig. 1.8 A). The cells enter mitosis and undergo EMT *(Leptin and Grunewald 1990; Bate et al., 1993).* The mesodermal cells migrate in dorso-lateral direction, following the leading edge cells, which form cellular

protrusions (Fig. 1.8 B). These protrusions disappear, when the cells reach their final positions and form a monolayer on the ectoderm (Fig. 1.8 C).

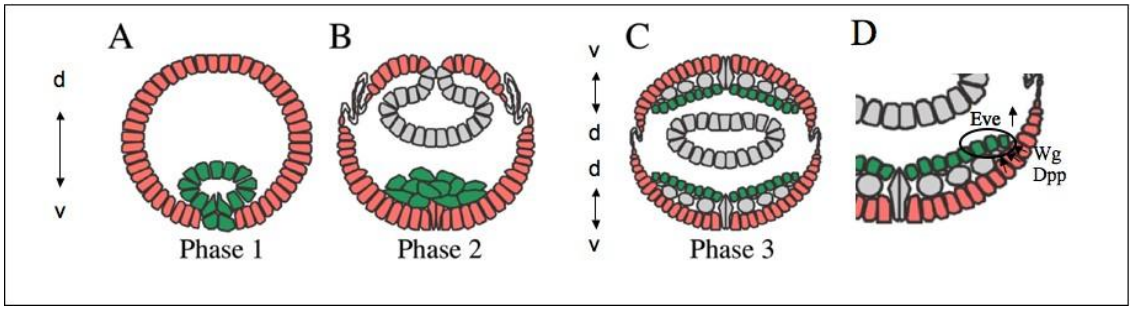


Fig. 1.8 Mesoderm development in *Drosophila.*
(A) The most ventral epithelial cells invaginate into the embryo and form a tube. The tube disassembles and the cells undergo EMT. (B) The cells form protrusions and migrate in dorsolateral direction. (C) The mesoderm cells (green) form a monolayer on the ectoderm (red). During germ band elongation there are 2 dorsal and 2 ventral sites in the embryo. The extension of the germ band runs in dorsal direction, because the size of the eggshell limits the elongation in posterior direction. (D) The mesoderm cells reach their final position and can receive Dpp and Wg signals, which are secreted by the ectoderm. The expression of Eve is initiated (modified after *Knust and Müller, 1998)*.

1.5.3 The specification of mesoderm derivatives

The mesoderm gives rise to heart, somatic muscles and other cell types in later organogenesis. During the development of the mesoderm the primordia of visceral, somatic and cardiac tissues are specified in each segment via signals from the ectoderm (Fig. 1.8 D). This is important for the determination of mesodermal cells to precursor cells of specific mesoderm derivates, like cardioblasts, pericardial cells and dorsal somatic muscles. *Dpp*, which belongs to the TGF β family and *Wg* the *Drosophila* Wnt homologue are two genes encoding for ectodermally secreted proteins. Dpp is a BMP orthologue and responsible for the determination of the visceral and somatic mesoderm derivates. Dpp signals cooperate with the mesoderm specific NK homeodomain transcription factor Tinman. Tinman is activated by Twi and also required for the initiation of mesoderm progenitor cells *(Lee and Frasch, 2005)*. Wg regulates the distribution of Dpp/Tin along the anterior-posterior axis required for the segregation of pericardial cells and muscles (Fig. 1.9) *(Bate et al., 1993; Frasch, 1995; Lee and*

Frasch, 2005). This happens by regulation of the expression of gene products in mesodermal cells.

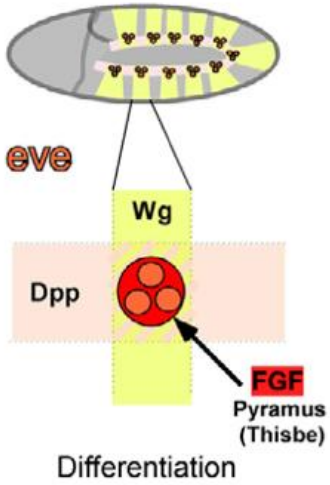


Fig. 1.9 Interplay between Wg, Dpp and FGF signalling leads to specification of mesoderm derivatives.
In a Drosophila embryo Wg is expressed in the hemisegments and regulates the expression of Dpp along the anterior-posterior axis. FGF receptor activation by Pyr and Ths is necessary to induce the expression of Eve and to specify the formation of pericardial precursor cells (*Kadam et al., 2009*).

The development of visceral mesoderm precursor cells is suppressed by Wg and induced by Dpp regulated through RTKs and FGF receptor Htl *(Carmena et al., 1998; Michelson et al., 1998; Englund et al., 2003).* For instance the pair rule gene even-skipped (Eve) is a homeobox gene expressed in the pericardial cells and muscle precursor cells *(Frasch et al., 1987)*. Eve expression in the hemisegments is regulated by Dpp, Wg and Htl (Fig. 1.9). In mutants that affect the migration, the formation of Eve positive pericardial cells is blocked. Therefore, the loss of these cells indicates defects in mesoderm migration.

1.5.4 Mesoderm spreading is regulated by the Heartless FGF signalling pathway

The FGF receptor Htl is required for cell migration and differentiation of the mesoderm *(Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998; Shishido et al., 1993; Shishido et al., 1997)*. In Htl mutants the mesodermal cells do not migrate and therefore are not at a position to receive the Dpp and Wg signals from the

ectoderm. As a result the mesoderm derivates are not induced and the embryo is lacking muscles and heart (*Schumacher et al., 2004; Michelson et al., 1998).*

Two ligands are known for the Htl FGF receptor, Thisbe (FGF8-like1) and Pyramus (FGF8-like2) *(Gryzik and Müller, 2004; Stathopoulos et al., 2004; Klingseisen et al., 2009).*

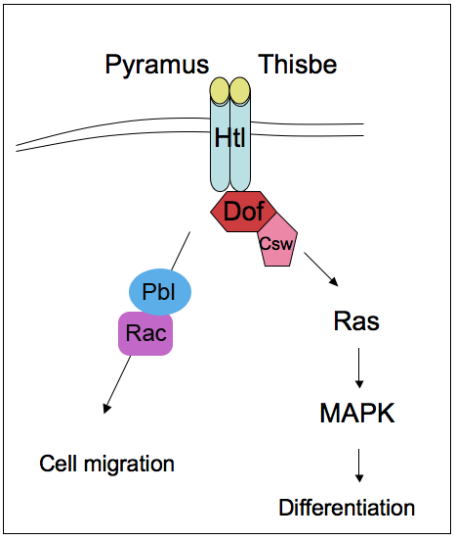


Fig. 1.10 Htl FGF receptor signalling pathway regulates cell migration and activation of MAPK.
The two ligands Pyramus and Thisbe activate Htl. Htl receptor phoyphorylates and activates the adaptor protein Dof, which recruits the phosphatase Csw. Both are required for the activation of MAPK cascade. Furthermore activation of Htl is required for the migration of the mesoderm cells.

Genetic analysis in our lab has shown that both ligands are sufficient and required to activate the Htl receptor. Pyr and Ths have overlapping functions during mesoderm spreading. However they show different expression patterns suggesting distinct functions during development. For instance Pyr is required for Eve expression in dorsal mesoderm derivatives whereas Ths is not *(Klingseisen et al., 2009).*

After binding of the two ligands to Htl and the formation of a complex with HSPGs, the adaptor protein Dof is activated and phosphorylated by the receptor (Fig. 1.10). Dof functions as a signalling mediator similar to the vertebrate FRS2 and is expressed only in tissues where the two *Drosophil*a FGF receptors Htl and Btl are expressed *(Vincent et al., 1998).* Upon activation Dof binds to the Tyrosin phosphatase Corkscrew (Csw) *(Petit et al., 2004; Wilson et al., 2004).* Csw recruitment to the Dof/FGF receptor complex is required for cell migration and MAPK activation in mesoderm and tracheal cell migration (Fig. 1.10) *(Johnson Hamlet and Perkins, 2001; Petit et al., 2004).* Csw is an orthologue of the vertebrate SHP-2 protein, SHP-2 is

required for the migration of the mesoderm cells away from the primitive streak via FGF signalling *(Saxton and Pawson, 1999);* furthermore SHP-2 is involved in branching morphogenesis in vertebrates *(Rosario and Birchmeier, 2003)*. Dof and Csw are required for activation of Ras1/MAPK cascade (Fig. 1.10). The activated form of MAPK, ERK is finally translocated into the nucleus where it activates and regulates and phosphorylates transcription factors, other kinases and cytoskeletal proteins.

The activation of MAPK seems to be required, but is not sufficient for mesoderm cell migration. In mutant embryos for the gene Pebble, which is a major regulator of mesoderm cell migration MAPK is activated, but the cells fail to migrate *(Schumacher et al., 2004; Wilson et al., 2005).* Moreover it was shown in tracheal cells that a truncated Dof protein can activate the RAS/MAPK, but the cells still do not migrate *(Petit et al., 2004).* On the other hand over-activation of MAPK interferes with migration in mesoderm and tracheal cells *(Petit et al., 2004; Klingseisen et al., 2009).* This indicates that Htl/Dof/Csw regulate an additional pathway parallel to Ras/MAPK cascade, which induces cell shape changes and migration via Pbl (Fig. 1.10).

Spry is an important regulator of MAPK; though the mechanisms of MAPK regulation via Spry are not well understood. In *Drosophila* a role for Spry antagonizing Btl FGF signalling in tracheal morphogenesis was described *(Hacohen et al., 1998).* Additionally Spry plays a role during glial cell migration in *Drosophila* by antagonizing Htl signalling *(Franzdottir et al., 2009).* However a role for Spry in Htl regulated mesoderm cell migration was not shown yet. Spry and Spred mutants do not exhibit defects in mesoderm migration. However they might have regundant functions during mesoderm development.

1.6 The Rho GEF Pebble (Pbl) is required for mesoderm migration

The gene *pbl* encodes a Rho Guanine nucleotide exchange factor for Rho GTPases. Pbl is required for the cell shape changes during mesoderm cell migration and cytokinesis in *Drosophila* embryogenesis *(Schumacher et al., 2004, Smalhorn et al., 2004*). It was shown that the functions of Pbl in cytokinesis and mesoderm development are independent (*Schumacher et al., 2004*).

Pbl is required for cell shape changes during cytokinesis

During cytokinesis Pbl and its human orthologue Ect2 regulate the adjustment of the contractile actin-myosin ring by activation of Rho1/RhoA *(Miki et al., 1993; Prokopenko et al., 1999)*. Rho1 is responsible for the assembly of actin-myosin contractile structure, which is important for the distribution of chromosomes, organelles and the cytoplasm of the daughter cells after mitosis *(Ridley and Hall., 1992)*.

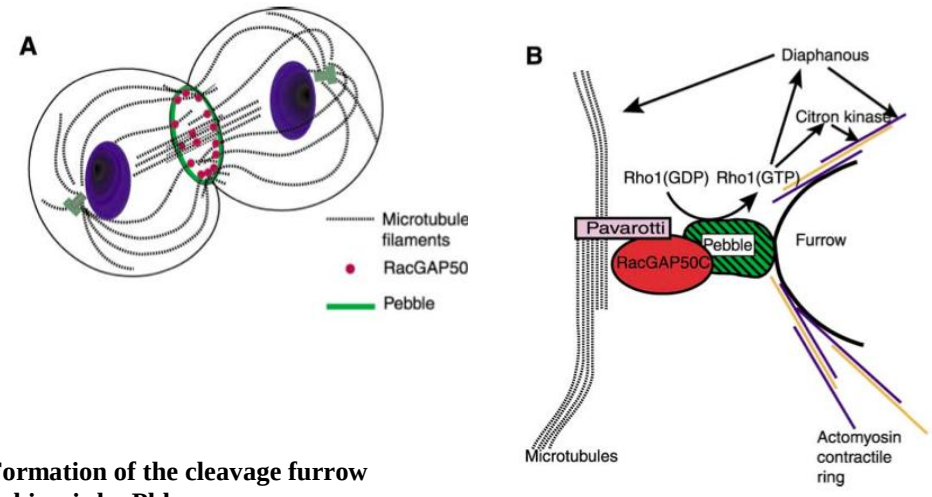


Fig. 1.11 Formation of the cleavage furrow during cytokinesis by Pbl.
RacGAP50C forms a complex with Pavarotti and recruits Pbl to the cleavage furrow. Pbl activates Rho1, who activates the Formin Diaphanous (Dia) and Citron Kinase, followed by actin filament assembly and the formation of an actin-myosin contractile ring *(Somers and Saint, 2003)*.

The RacGAP50C/Cyk-4 and the kinesin-like protein Pavarotti form a complex, so called centralspindlin complex and are required for the bundling of the microtubules (Fig. 1.11). Pbl directly interacts with its N-terminal BRCT domains with RacGAP50C *(Somers and Saint, 2003; Simon et al., 2008)* and is recruited to the cleavage furrow *(Wolfe et al., 2009)* (Fig. 1.11). At the celavage furrow Pbl activates Rho, inducing a

formation of the actin-myosin contractile ring. This happens via activation of Diaphanous (Dia) that is required for actin filament assembly and the activation of Citron Kinase and Myosin II (Fig. 1.11) *(Prokopenko et al., 1999; Shandala et al., 2004; Hickson and O'Farell, 2008)*. Other proteins like Anilin and Septins are relocated to the complex and link the contractile furrow elements to the plasma membrane and to microtubles *(Hickson and O'Farell, 2008)*.

Mutations in both Pbl and Ect2 result in a failure of cytokinesis and consequent in multinuclear cells (Fig. 1.12).

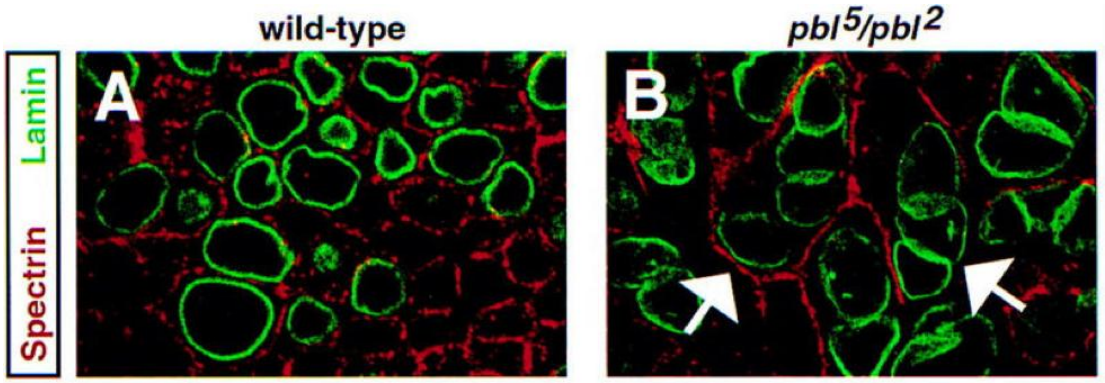


Fig. 1.12 Cytokinesis defects in *pbl* mutants.
Embryos stained with anti-α-spectrin (red) to mark the plasma membrane and anti-laminin (green) to mark the nuclei. (A) In wild type embryos one nucleus is found per cell. (B) in *pbl* mutants several nuclei are surrounded by one plasma mebrane in one cell (arrows) (*Prokopenko et al., 1999*).

Pbl is required for cell shape changes during mesoderm migration

Our laboratory and others have found that Pbl has an important function during mesoderm migration (*Gryzik. and Müller, 2004; Schumacher et al., 2004; Smallhorn et al., 2004).* This becomes apparent in *pbl* mutants, where the mesodermal cells fail to change their cell shapes, do not form protrusions and fail to migrate. The mesoderm does not spread in dorsolateral direction (Fig. 1.13 A, B, C). The formation of Eve expressing pericardial cells in each segment fails (Fig. 1.13 E). It was suggested that the defects during cell migration are a result of the defects in cytokinesis. Therefore the mesoderm migration phenotype of Pbl was analyzed in the background of a *string* (*stg*) mutant. In *stg* mutants the cell cycle is blocked in G2/M phase transition, consequently

the cells do not go into mitosis (*Edgar and O'Farrell, 1989)*. In these mutants the mesodermal cells form cellular protrusions, migrate and mesoderm specification proceeds as in wild type. In *stg, pbl* double mutant embryos cell migration during mesoderm development is affected. For the reason that the cells arrest before they go into mitosis an effect of the cytokinesis defects of *pbl* on the migration defects can be excluded *(Schumacher et al., 2004)*. The function of Pbl in mesoderm migration is independent of the function in cytokinesis.

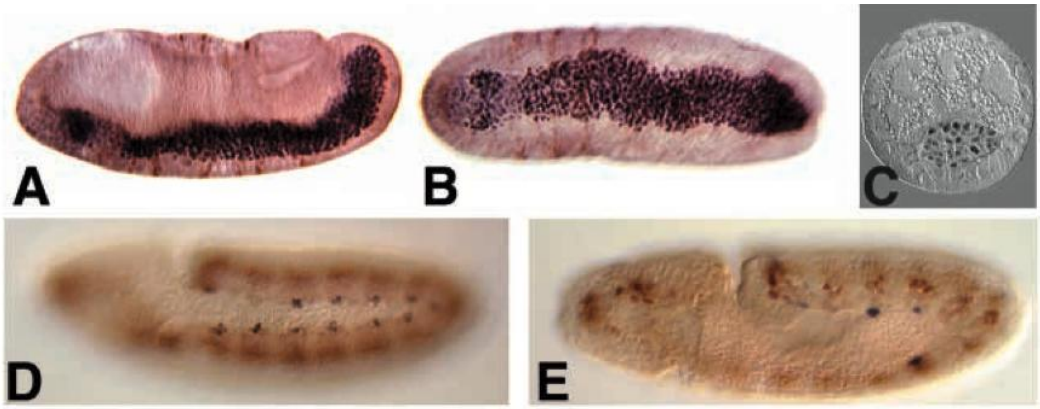


Fig. 1.13 The mesodermal cells fail to migrate in *pbl* mutants.
(A) Lateral and (B) ventral view of a *pbl* mutant embryo. The mesodermal cells are stained with α Twi antibody. They fail to migrate in dorsalateral direction. (C) Cross-section of a *pbl* mutant embryo, the cells do not migrate. (D) Lateral view of a wild type embryo. 11 Eve positive cells are marked with α Eve antibody. (E) Only two Eve positive cell cluster are visible in *pbl* mutants (*Schumacher et al., 2004*).

The function of Pbl during cytokinesis is well described, whereas the mechanisms upstream and downstream of Pbl during mesoderm cell migration are not well understood. RhoA is not the substrate of Pbl during mesoderm migration, since the expression of $RhoA^{N19}$, a dominant negative variant of Rho1 does not affect mesoderm migration but does block Rho1 dependent cytokinesis *(Schumacher et la., 2004).* During mesoderm migration Pbl shows exchange activity for Rac and activates it by direct interaction. Furthermore *rac* mutant embryos exhibit severe defects in mesoderm spreading *(van Impel et al., 2009).*

The protein structure of Pbl

Pbl belongs to the Dbl homology protein family of GEFs. In the C-terminal region Pbl contains the DH-PH tandem domain, the catalytic entity of the protein. In the central region Pbl contains a NLS (nuclear localization signal) domain that is responsible for its localization to the nucleus and a PEST domain, an amino acid-motif, enriched in Proline (**P**), Glutamate (**E**) Serine (**S**) and Threonine (**T**), which is recognized by ubiquitin ligases for the degradation of the protein by the proteasome. Pbl is regulated by the ubiquitin ligase UBE3A, which is responsible for Angelman syndrome (AS) in human *(Reiter et al., 2006)*. In the N-terminal region there are two BRCT domains, which are protein-protein interaction domains *(Bork et al., 1997)* that are important for the localization of Pbl at the cleavage furrow during cytokinesis *(Somers and Saint, 2003)*.

Fig. 1.14 Protein structure of Pbl.

In addition Pbl contains an evolutionary conserved C-terminal tail. Structure function analysis has shown that the C-terminal tail of Pbl might be required for the Pbl localization and function *(van Impel et al., 2009)*. A truncated protein encoded by the $Pbl^{DH\text{-}PH}$ transgene, containing only the catalytic domains DH and PH, is a constitutive active from of Pbl. Expression of $Pbl^{DH\text{-}PH}$ in wild type embryos leads to migration defects, similar to the defects observed in *pbl* loss of function mutants. The over-expression of a transgene, $Pbl^{\Delta N\text{-}term}$ which contains the DH, the PH domain and the C-terminal tail leads to defects during invagination, a process regulated by Rho1, but not by Pbl. Furthermore the $Pbl^{DH\text{-}PH}$ construct rescues the mesoderm phenotype in *pbl* mutants quite well, whereas the second construct $Pbl^{\Delta N\text{-}term}$, which contains the C-terminal tail fails to rescue the migration defects. This indicates that the C-terminal tail

might have a function to regulate the substrate specificity of Pbl for Rho1 and Rac *(van Impel et al., 2009).* Indeed it was shown for Ect2 in cultured mammalian cells that the C-terminal tail is phosphorylated. For that reason it was proposed, that phosphorylation of the C-terminal tail is responsible for the substrate specificity or needed for the regulation of Ect2. In fact in a phosphorylation deficient mutant of Ect2 the activation of RhoA is reduced *(Niiya et al., 2006).*

According to the function of Pbl in the reorganization of the actin cytoskeleton and the similar phenotype to *htl* mutants, Pbl could be linked to the FGF signalling pathway. It was indeed shown, that the activity of Pbl is required for the function of Htl in the regulation of cell shape changes. The expression of Htl and λHtl, a constitutive active form of the receptor, were unable to induce cell shape changes in early *pbl* mutant embryos. A partial rescue could be observed in later mesoderm development in *pbl* mutants by expression of λHtl. These results show that Pbl is required for the early cell shape changes triggered by Htl, but there are additional mechanisms necessary for spreading of the mesoderm. Furthermore it was shown that in *pbl* mutant embryos the MAPK is still activated. MAPK activation in response to Htl is unaffected. This suggests that the MAPK alone is not sufficient for cell shape changes in mesoderm migration, but there might be a different parallel signalling pathway involved in mesoderm development *(Schumacher et al., 2004).*

1.7 Aims of this study

According to the important function of Pbl in mesoderm migration by triggering cell shape changes and the formation of protrusions, two questions were raised. How is Pbl linked to the FGF signalling pathway? And how is the localization and activity of Pbl regulated in cell migration?

Two experimental approaches will be used to address these questions. The first approach is a genetic screen to find new interactors of Pbl, which might give some insight into the function of Pbl by identifying interacting proteins. The screen is based on the gain of function rough eye assay. Over-expression of $Pbl^{DH\text{-}PH}$ in the eye of *Drosophila* leads to strong defects in the shape of the ommatidia. By overexpression of this transgene, in flies who have either chromosomal deletions or point mutations in particular genes, modification of these defects can be analyzed. Therefore the candidates might be interactors of Pbl and will be characterized and analyzed regarding to their function in mesoderm cell migration.

A second approach is a structure function analysis of Pbl, which addresses the regulation and localization of Pbl. It will be investigated if the C-terminal tail is essential for the function of Pbl. Furthermore analysis in our lab has shown that the PH domain plays an important role in the activity of Pbls DH domain. Both domains might be important for the localization of Pbl as well as for the binding and regulation by other proteins. With transgenes that are lacking the PH domain and the C-terminal tail the function and localization of these will be analyzed. If an essential influence of the C-terminal tail on the function of Pbl can be shown, a potential phosphorylation site of the C-terminal tail will be examined.

The aim of this thesis is to find interactors of Pbl by using a genetic screen and a structure function analysis. The results will give an insight into how Pbl regulates cell shape changes during mesoderm development and how it is linked to the FGF signalling pathway.

2 Methods and Materials

2.1 Materials and Equipment

2.1.1 Chemicals

The Chemicals were purchased *pro analysis* by the following companies:

Aldrich, Steinheim; Amersham, Braunschweig; Applichem, Darmstadt; Baker, Deventer,

Niederlande; Biomol, Hamburg; Bio-Rad, München; Biozym, Hameln; Difco, Detroit, USA; Fluka, Neu-Ulm; Gibco/BRL Life Technologies, Karlsruhe; Merck, Darmstadt; Pharmacia, Freiburg; Roth, Karlsruhe; Riedel-de Haen, Seelze; Roche Diagnostics GmbH, Mannheim; Sigma-Aldrich, Steinheim.

All solutions were prepared with autoclaved dH_2O.

The Restrictionenzymes were purchased from *Boehringer/Roche Diagnostics,* Mannheim Germany; *Promega*, Madison, USA; *MBI Fermentas*, St. Leon-Rot, Germany.

For DNA extraction, purification and isolation the following kits were used:

- NucleoSpin Extract, *Macherey Nagel*, Düren, Germany
- QIAquick Gel Extraction Kit, *Qiagen*, Hilden, Germany
- Qiagen Plasmid Midi Kit, *Qiagen*, Hilden, Germany
- Nucleobond AX, *Macherey Nagel*, Düren, Germany
- TOPO TA Cloning Kit, *Invitrogen*, Groningen, Netherlands

2.1.2 Microscopy, Equipment and Software

Microscopy was performed on a Leica-SP2 confocal microscope (*Leica*, Heidelberg Germany) and Olympus BX61 (*Olympus*, Watford, UK).

Images were processed using Adobe Photoshop (*Adobe Systems*, San Jose, USA), Volocity (*Improvision, a PerkinElmer Company*, Coventry, UK)

For sequence analysis DNA-Star Lasergene V6 (*DNASTAR Inc.*, Madison, USA) was used on a Macintosh system (*Apple*, Ismaning, Germany).

2.1.3 Vectors

The created transgenes were sub-cloned into pBSKII (Stratagene) and then finally into pUAST-HA *Drosophila* expression vector (*Perrin, 2003* #278).

2.1.4 Oligonucleotides

The following Oligonucleotides were used for PCR:

Name	Sequence 5'-3'	Binding	Purpose
358069	CGGAATTCGTAACTGCAGAAGATCCATG	5' to first ATG	all constructs
3'Pbl	CGGAATTCCGAATGCGGCCCACAACGGCC	3' to coding sequence	all constructs
3'bfPH	GCTCTAGAGCCGATTCGGTCCGCCTTTTATC	after the DH domain	Pbl $^{\Delta PH\text{-}HA}$
5'aPH	GCATCTAGAGCCGGCAGCCCACACCTGC	after the PH domain	Pbl $^{\Delta PH\text{-}HA}$
S-A 5'	GCAAGCATGCGAAGCAGTGCTCCGTCAACACAATCCG	before and after Serine 825	Pbl ^{S825A}
S-A 3'	CGGATTGTGTTGACGGAGCACTGCTTCGCATGCTTGC	before and after Serine 825	Pbl ^{S825A}

Table 2.1. Oligonucleotides

2.2 Molecular Biology

2.2.1 PCR

The Polymerase-Chain-Reaction (PCR) is a common method to isolate and amplify single genes of the genome (*Mullis and Faloona, 1987*). A thermostable enzyme Taq polymerase, which is segregated from the bacteria *Thermus aquaticus* is able to synthesize high amounts of DNA. To avoid mis-synthesis another enzyme the Pfu-Polymerase is used, which a proofreading function. The starting point for the synthesis of DNA are oligonucleotides, so called primer, which are complementary to both DNA strands. The DNA is amplified in repeating cycles of Denaturation of the DNA, Annealing of the primer and Synthesis of the DNA.

An example for a PCR reaction mix in 50µl volume is shown below:

- Xµl DNA (50ng)
- 10µl Pfu-Polymerase buffer (5x)
- 0,5µl forward primer (50µm)
- 0,5µl reverse primer
- 4 µl dNTP's (25mm)
- 1µl Pfu-Polymerase
- X µl dH_2O

	duration	temperature	step
	5min	94°C	Denaturation
1	30s	94°C	Denaturation
2	30s	58°C (primer dependent)	Annealing
3	Y (dependent of the product 1Kb/min)	72°C	Elongation
repeat 1,2 and 3 x 34 cycles			
	5min	72°C	finale elongation

Table 2.2 Standard PCR

The PCR product was tested by Agarose-gel Electrophoresis. Additionally when the PCR product was used for cloning it was purified with the Wizard clean-up (Promega).

2.2.2 Site directed Mutagenesis

For mutation of specific sites in the DNA primer were designed, which contain the designated basepair exchanges. The template for the mutagenesis was the Pbl EST clone SD01796. With the Quick Change Site Directed Mutagenesis kit (Stratagene) the mutagenesis reaction was performed as described in the handbook.

PCR reaction mix in 50µl

- 5µl DNA
- 5µl reaction buffer (10x)
- 1µl dNTP's
- 0,6 µl primer A (S-A5' +3' 125ng)
- or 0,7µl primer B (S-H 5'+3' 125ng)
- xµl dH_2O

The PCR product was cut with the endonuclease DpnI for 1h 37C. Afterwards 1µl was transformed into 50µl DH5α cells and plated on agar plates that containing Chloramphenecol. The colonies were tested by sequencing.

The DNA with the exchanged sequences was used for PCR with primers that contain EcoRI sites. The PCR products were cleaved with EcoRI and ligated into the expression vector pUAST-HA.

2.2.3 Cloning of PCR products into plasmid vectors

To get high amounts of DNA the generated constructs can be transformed into cells that synthesize the transgene. For that purpose the PCR product is ligated into a Vector that is transformed into *E.coli* cells. Afterwards the DNA can be isolated out of the cells.

Ligation

To bring a construct into a vector, both were cleaved with endonucleases and purified with Wizard clean-up (Promega).

- 1µl Vector (1-2µg)
- 1,5 µl Ligase buffer
- 1µl Ligase
- 12µl DNA (5x more than vector)

The Ligation was carried out overnight at 16°C.

Transformation of chemo-competent cells

The cells that were used for the transformation are DH5 and XL-10 cells depending on the antibiotic resistance of the vector.

- 7µl-15µl of the ligation were added to 50µl of cells
- incubate on ice for 30 min
- heatshock on 42°C for 30-40 sec
- put on ice for 2 min
- add 450 µl of LB- medium
- incubate for 45min on 37°C
- plate 200 µl of cells on LB+antibiotics plate
- incubate overnight on 37°C

Overnight colonies were picked and put into vials with 2ml of LB+amp medium to get higher amounts of cells. The vials were incubated overnight on 37°C.

Isolation of DNA, Preparation of Plasmid DNA

After transformation of a construct into a plasmid, the amplified DNA will be isolated from the cell colonies by a so called ‘Mini Prep’ using the Mini DNA purification kit from Qiagen. For synthesis of a higher DNA amount a bigger cell culture, Midi or Maxi Prep are used.

2.2.4 DNA cleavage using endonucleases

DNA cleavage of PCR products

To clone a construct into a vector the ends of the construct were cut with restriction enzymes so called endonucleases. Therefore primers were designed that contain restriction sites. An example for a digestion mix is given below:

- 30µl PCR-Product
- 5µl 10x reaction buffer
- 2µl Enzyme A
- 2µl Enzyme B
- 11µl HO

The reaction was carried out for 1-2h on 37 °C (dependent on the endonuclease).

The vector was cut with the same enzymes. To prevent re-ligation of the two ends of the vector these ends were dephosphorylized. Therefore 1µl of Antarctic phosphatase was added to the reaction mix for 15min. The phosphatase removes the phosphates at the 5' end of the vector and the ends of the vector cannot religate.

Test cleavage after cloning

- 10µl Mini-DNA respecively 1µg Midi-DNA
- 2µl 10x reaction buffer
- 0,5µl Enzyme A
- 0,5µl Enzyme B
- 7µl H_2O

The reaction is carried out for 1-2h on 37 °C (dependent on the endonuclease).

2.3 Genetic methods

2.3.1 *Drosophila* breeding and maintenance of stocks

The flies were kept on 18°C in vials containing standard medium. Crosses were kept on 25°C, to push the development. To collect embryos the flies were put into cups, which were placed on agar plates containing apple juice and fresh yeast to stimulate egg laying.

Standard food medium: 356g maize coarse meal, 47.5g soy flour, 84g dry yeast, 225g malt extract, 75ml 10% Nipagine, 22.5ml propionic acid, 28g agar, 200g sugar beet molasses, 4.9L dH2O

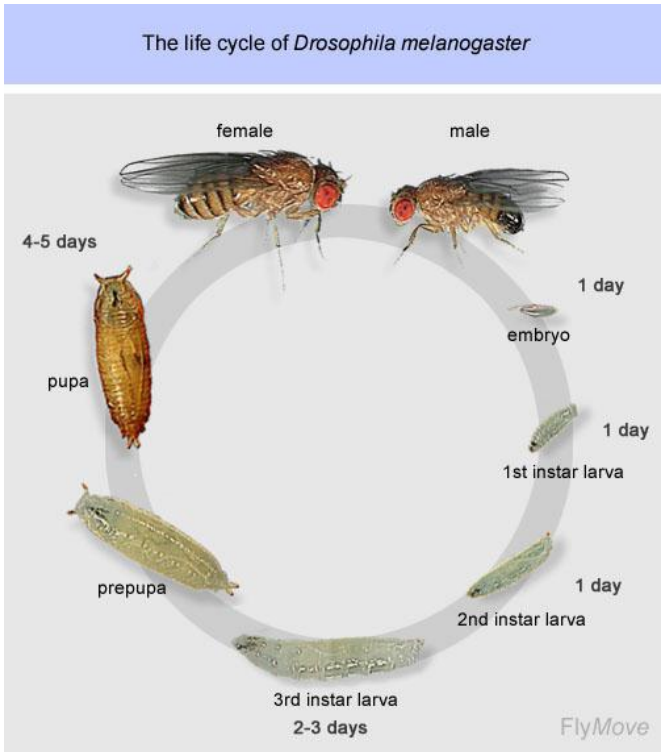


Fig. 2.1 Life cycle of *Drosophila melanogaster*
The development of Drosophila takes 10 days on 25°C. After an embryo is fertilized, the embryogenesis lasts 24h and the first instar larvae hatch. The first and second larval stages last for one day each. The third instar larvae develops for 3 days and goes into pupal stage. After 4-5 days the adult fly hatches. The adult flies are fertile 4-8 hours after eclosion (depending on the temperature) (http://flymove.uni-muenster.de).

Drosophila melanogaster is a holometabolic insect, which means the development includes metamorphosis. The generation from embryo to adult fly takes 10 days at 25°C. At 18°C the life cycle is slower and takes 20 days. The development of

the embryo last for 24h then the first instar larvae (L1) develops. The generation of L1 and L2 each last for one day. The development of the third instar larvae (L3) takes 2-3 days, then the L3 moves to a solid surface and goes into pupal stage. The metamorphosis takes 4-5 days and only a few hours after the flies eclosed they become fertile.

2.3.2 Fly Stocks

The fly stocks used in this thesis were either purchased from Bloomington and from different sources as mentioned. The transgenic flies were either generated by germline-transformation or send away for injection to Rainbow Transgenic Flies, Inc. (*Newbury Park, CA 91320 USA; rainbowgene.com*).

Mutations

Name	Chromosome	Function	Reference
w1118	1	mutation in the *white* gene, used for germline-transformation	Lindsley and Zimm, 1992
htl[$^{AB42]}$ / TM3 $^{[ftz::lacZ]}$	3	*htl* loss of function mutation	Alan Michelson
htl$^{[YY262]}$ / TM3 $^{[ftz::lacZ]}$	3	*htl* hypomorph mutation	Alan Michelson
IF/CyO; *pbl*^{3} *e* /TM3$^{[ftz::lacZ]}$	2+3	*pbl* loss of function mutation; markers and balancer on the 2. + 3. Chr.	Arno Muller

Table 2.3. Fly stocks carrying mutations

Driver-/Activator- lines

Name	Chromosome	Function	Reference
twi::Gal4	2	driver of expression in the mesoderm	Gerold Schubiger
twi::Gal4, twi::CD2	2	Mesoderm driver on the 2nd chromosome and CD2 expression in the mesoderm	Bloomington stock centre
w; *twi::Gal4* /CyO; *htl*[$^{AB42]}$/TM3$^{[ftz::lacZ]}$	2+3	Mesoderm driver on the 2nd and *htl*	Arno Muller

		mutation on the 3rd	
w; *twi::Gal4* /(CyO) ; *pbl*^{3}/TM3$^{[ftz::lacZ]}$	2+3	Mesoderm driver on the 2nd and *pbl* mutation on the 3rd	Andreas van Impel

Table. 2.4. Driver-lines

Effector-/Responder-lines

Name	Chromosome	Function	Reference
UAS::Pbl $^{DH\text{-}PH\text{-}HA}$	2	HA tagged, constitutive active Pbl construct	Andreas van Impel
GMR::G4, UAS::Pbl $^{DH\text{-}PH\text{-}HA}$	2	HA tagged, constitutive active Pbl construct expressed in the eye	Andreas van Impel
UAS::Pbl$^{\Delta PH\text{-}HA}$	2	HA tagged Pbl construct	this work
UAS::Pbl $^{\Delta Cterm\text{-}HA}$	2	HA tagged Pbl construct	this work
UAS::Pbl $^{S825A\text{-}HA}$	2	HA tagged Pbl construct	this work
Pbl$^{\Delta PH\text{-}HA}$; *pbl*^{3}/TM3$^{[ftz::lacZ]}$	2+3	HA tagged Pbl construct and *pbl* mutation	this work
Pbl$^{\Delta Cterm\text{-}HA}$; *pbl*^{3}/TM3$^{[ftz::lacZ]}$	2+3	HA tagged Pbl construct and *pbl* mutation	this work
Pbl$^{S825A\text{-}HA}$; *pbl*^{3}/TM3$^{[ftz::lacZ]}$	2+3	HA tagged Pbl construct and *pbl* mutation	this work
Pbl$^{C\text{-}term\text{-}HA}$/CyO; *htl*^{AB42}/TM3$^{[ftz::lacZ]}$	2+3	HA tagged Pbl construct and *htl* mutation	this work
Pbl$^{DH\text{-}PH\text{-}HA}$/CyO; *htl*^{AB42}/TM3$^{[ftz::lacZ]}$	2+3	HA tagged Pbl construct and *htl* mutation	this work

Table 2.5. Activator-lines

Balancer chromosomes

Balancer chromosomes are a commonly used tool in *Drosophila* genetics to balance homozygous lethal mutations and deletions. The balancer is a chromosome, containing repeated sequences to avoid recombination with the chromosome that carries the mutation, so the mutation is kept heterozygous stable. Furthermore balancer

chromosomes contain dominant mutations, which are used as markers to track the chromosome and are homozygous lethal.

Name	Chromosome	Function	Reference
IF/CyO^{ftzlacZ}	2	Balancer for the 2. and 3. Chromosome with βGal marker	Arno Müller
Gla/CyO^{ftzlacZ}	2	Balancer for the 2. Chromosome βGal marker	Tanja Gryzik
TM3^{ftzlacZ}/TM6	3	Balancer for the 3. Chromosome βGal marker	Kevin Johnson
MKRS	3	Balancer for the 2. and 3. Chromosome	Lindsley und Zimm, 1992

Table 2.6. Balancer chromosomes

Suppressor mutants generated with EMS

Name	Genotype	
FRT second chromosome	FRT40	original stocks that were mutagenized with EMS, Bloomington
FRT third chromosome	FRT2A FRT82B/TM3	
Su (3) 18	*mut*/TM3 ^{lacz}	
Su (3) 29	*mut*/TM3 ^{lacz}	
Su (3) 21-10-3	*mut*/TM3 ^{lacz}	
Su (3) 21-10-4	*mut*/TM3 ^{lacz}	
Su (3) 21-10-30	*mut*/TM3 ^{lacz}	
Su (3) 26-10-3	*mut*/TM3 ^{lacz}	
Su (3) 26-10-27	*mut*/TM3 ^{lacz}	
Su (3) 26-10-31	*mut*/TM3 ^{lacz}	
Su (3) 28-10-3	*mut*/TM3 ^{lacz}	

Su (3) 28-10-8	*mut*/TM3 ^{lacz}
Su (3) 29-10-3	*mut*/TM3 ^{lacz}
Su (3) 29-10-7	*mut*/TM3 ^{lacz}
Su (3) 31-10-1	*mut*/TM3 ^{lacz}
Su (3) 3-11-4	*mut*/TM3 ^{lacz}
Su (3) 3-11-53	*mut*/TM3 ^{lacz}
Su (3) 10-11-1	*mut*/TM3 ^{lacz}
Su (3) 10-11-14	*mut*/TM3 ^{lacz}
Su (3) 17-12-2	*mut*/TM3 ^{lacz}
Su (3) 17-12-2	*mut*/TM3 ^{lacz}
Su (3) 25-12-4	*mut*/TM3 ^{lacz}
Su (2) 27-4-5	*mut/CyO*
Su (2) 27-4-10	*mut/CyO*
Su (2) 20-5-6	*mut/CyO*

Table 2.7 EMS induced mutants used in the modifier screen

The flies used for the deletion modifier screen were all purchased from Bloomington and a list of the lines is in the appendix.

2.3.3 UAS/Gal4 System

The UAS/GAL4 System is a method to express genes ectopically at a specific time point (*Brand and Perrimon, 1993*). Two different transgenic flies are needed for the ectopic expression of a transgene. There is the activator- or driver- line, which contains a transgene that encodes the transcription factor Gal4, from the yeast under control of any enhancer. Furthermore an effector-/responder-line is used, which contains the transgene of interest behind an upstream activating sequence (UAS). UAS

is a regulatory element from the yeast, which can bind to Gal4 protein. The binding of Gal4 to the UAS induces the expression of the transgene.

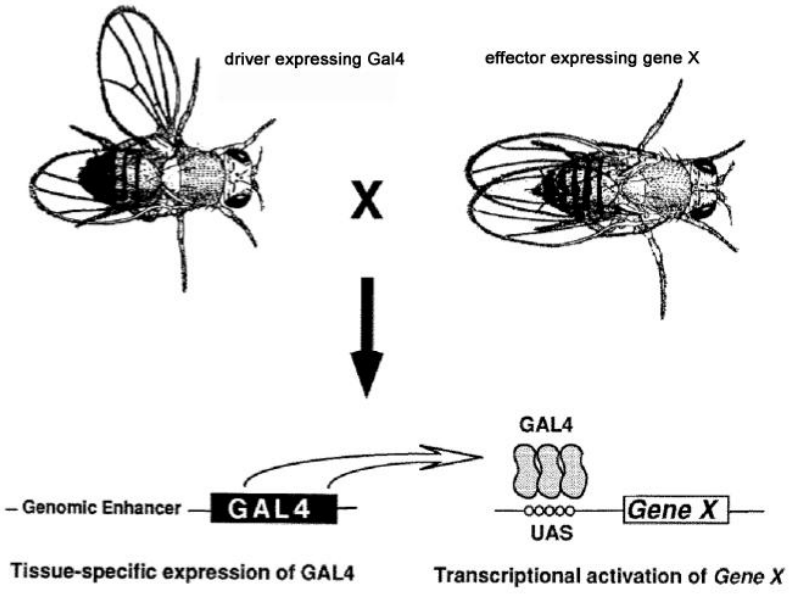


Fig. 2.2 UAS/Gal4 system.
The driver line encodes the Gal4 transgene under a tissue specific promotor. The effector line contains the transgene of interest (gene X). After crossing flies with both transgenes the progeny expresses both. Gal4 is expressed tissue specifically and binds to the UAS sequences, which induces the expression of the transgene X (*Brand and Perrimon, 1993*).

2.3.4 Germline-transformation

The transformation of the germline of *Drosophila* is a commonly used method to establish transgenic flies (*Spradling, 1986*). Transposable elements, so called P-elements are used for the gene transfer. The P-elements are flanked with so called inverted repeats, these are recognized and mobilized by transposase. For the germline transformation the DNA of the transgene is cloned into a vector that contains the inverted repeats, a second vector is used containing the Δ2-3 transposase (*Laski et al., 1986*). Both are injected into the embryo and the transposase can induce the mobilization and insertion of the transgene into the genome.

Injection

For injection embryos were used, which have a mutation in the *white (w)* gene and do not express eye color. The plasmid, were the trangene is cloned in, contains a wild type copy of the *w* gene. Flies express the red eye colour (it can differ between a range of yellow to red) when the transgene was integrated into the genome and can be detected easily.

Embryos were injected in the first 1.5 hours after the eggs were laid, when the embryo exist as a syncitium (*Campos-Ortega and Hartenstein, 1997*). It is important to inject the syncitial embryos into the posterior part of the embryo before cellularization occurs, so that the injected DNA can be integrated into the pole cells during cellularization.

The flies were put into cups on apple juice plates. It is recommended to prepare the flies 2-3 prior to injection, so that the flies can get used to their environment. On the day of injection the apple juice agar plates were changed every 30min. The eggs were collected and dechorionized with NaHCl. The embryos were aligned in one row on a small piece of apple juice-agar and then sticked to a cover slip. The embryos needed to dry for 5-7 minutes, otherwise they would burst when the needle went in. When they form wrinkles after touching them softly with a preparation needle the embryos are ready for injection. The embryos were covert with oil and the cover slip placed on a slide. Injection was carried out with a micromanipulator and was visually controlled with a misroscope. The injected embryos were covert with oil and put into a wet chamber at 18°C. After 2 days first instar larvae were collected and put into a food vial.

Injectionmix

Both plasmids were isolated with the Quiagen DNA midi kit after cloning.

- 7,5µl DNA (insert and pUAST, 600ng)

- 1µl Δ2-3 plasmid
- 1,5µl dH2O

The germline-transformation was performed for the *UAS:: Pbl$^{\Delta PH}$* transgene. The other transgenic lines were generated by Rainbow Transgenic Flies, Inc. (*Newbury Park, CA 91320 USA; rainbowgene.com*).

Balancing and Outcrossing

It is not possible to tell whether the injected flies carry the transgene inserted into the genome. Therefore the flies are crossed to w^- flies. In the next generation flies hatch that have red eyes, these are the flies with successfully intergration of the transgene into the genome. These flies were collected and crossed to flies that carry balancer chromosomes. Stable stocks were established with insertions on the second chromosome.

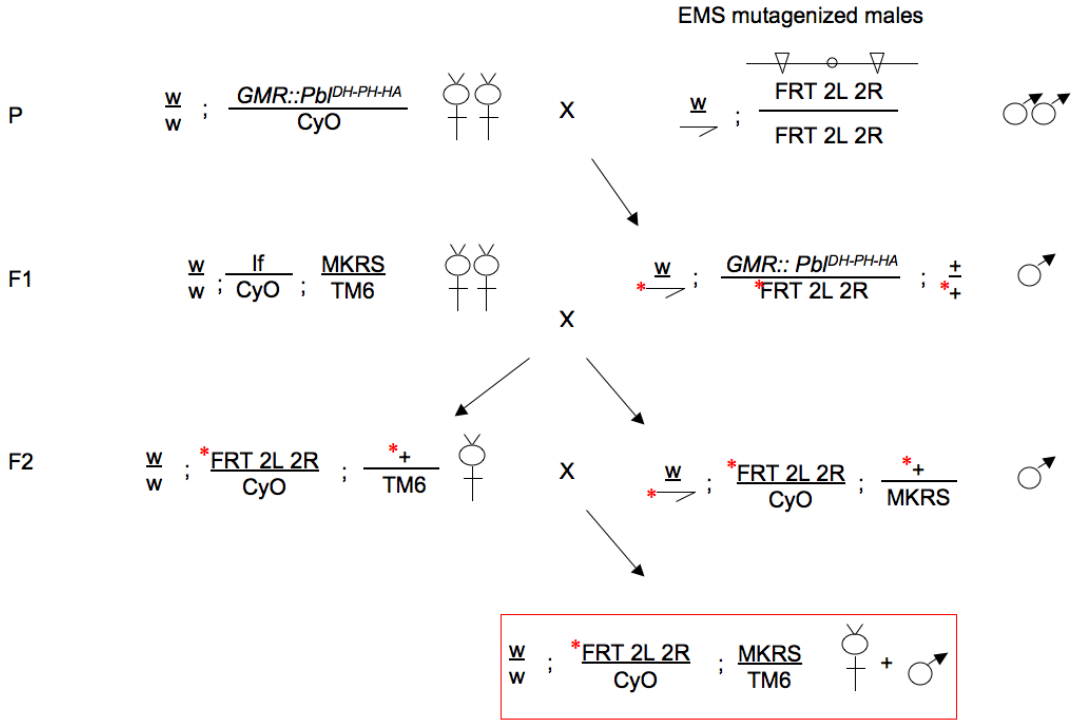
2.3.5 EMS mutagenesis

Ethyl methane sulfonate is an alkylating agent. EMS alkylates guanine and thymine which allows mispairing of both and leads to pointmutations. The procedure was modified after a protocol of T. A. Grigliatti (1986).

Male flies were collected three days after hatching. They were put on vials without food, only a small drop of water. After starving over night the males were put onto vials containing a sucrose-EMS solution. 100ml of 1% sucrose solution were prepared. A 25mM EMS solution was made adding EMS into the sucrose. Three pieces of whatman filter paper were placed on the bottom of the vials. With a 5ml syringe 3-4 droplets of EMS-sucrose solution were put on the filter paper, so that the paper is soaked with the solution but not too wet. Then 50 males, which were collected the day before and

starved over night were put into the bottles, carefully so that they do not stick to the EMS sucrose solution. After 24h the flies were removed from the vials and put on fresh vials with their normal food. They ate enough of the EMS during that time to induce pointmutations in the genome, longer exposure to EMS could lead to death. Swapping them another 1 or 2 times onto fresh food vials allows them to remove the EMS from their feed. Then the mutagenized males can be crossed to females, which carry the *Pbl* $^{DH\text{-}PH}$ transgene. The crossing schemes are displayed below.

Crossing scheme for the mutations on the second chromosome:



Crossing scheme for the mutations on the third chromosome:

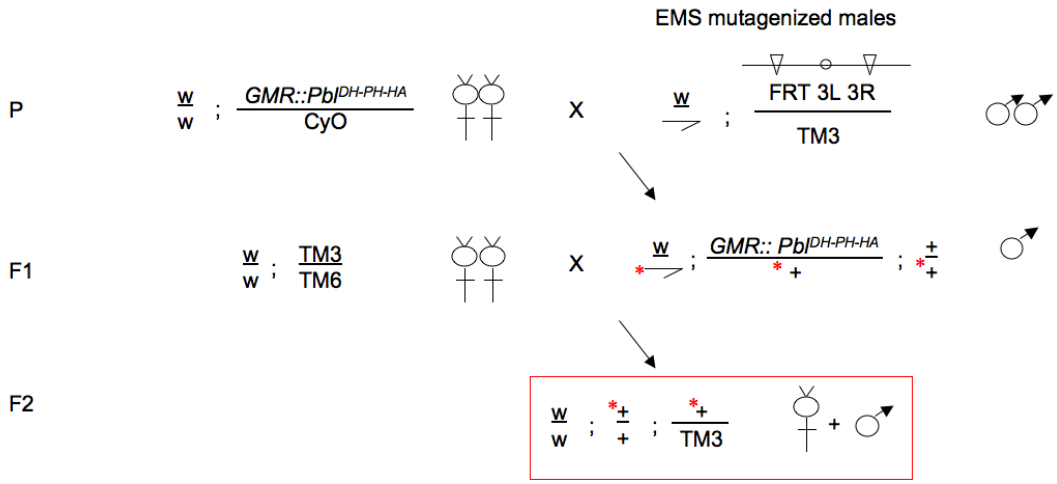


Fig. 2.3 Chrossing scheme for mutations on the second and third chromosome.
Mutagenized males were crossed with females expressing Pbl DH-PH in the eye. The F1 generation was screen for rough eye modification and crossed to balancer flies. F2 stable stocks were established.

2.3.6 Generation of germline mosaics

The production of germ line mosaics is a standard genetic tool in *Drosophila*. Heterozygous females carrying a mutation (a^{+}/a^{mut}) are crossed with heterozygous males (a^{+}/a^{mut}). The progeny is either heterozygous like the parents (a^{+}/ a^{mut}), homozygous wild type (a^{+}/a^{+}) or homozygous mutant (a^{mut} / a^{mut}). However the homozygous mutant embryos still carry the maternal gene product. To produce homozygously mutant germ cells, mitotic recombination is experimentally induced in a heterozygous germ line stem-cell. Thereafter the arising cystoblast is homozygous for the mutation and thus all the 16 germ cells including the oocyte developing out of this cystoblast are homozygous for the mutation and finally do not produce any gene product. The fertilization of these oocytes by sperm of heterozygous males, results in two types of zygotes. On the one hand embryos develop, which are carrying no maternal gene product but a functional zygotic copy of the gene (a^{mut} /a^{+}) and on the other hand embryos, which do not have any functional gene product, neither maternal nor zygotic (a^{mut} / a^{mut}) and are therefore also referred to maternal-zygotic mutants (*Janning and Knust, 2004*).

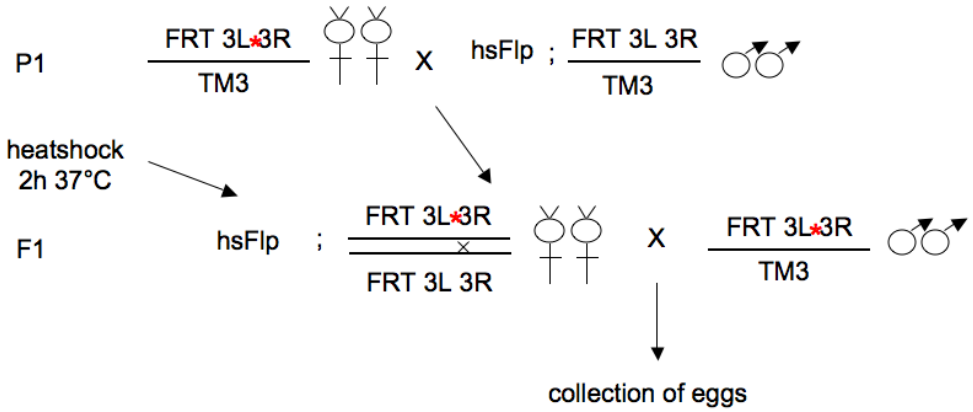


Fig. 2.4 Crossing scheme for the generation of germline clones.

Nevertheless a differentiation between embryos derived from a homozygous germ cell and embryos derived from a heterozygous germ cell is required. For that reason the dominant female sterile mutation Ovo^D is used (*Busson et al., 1983*), which is on the same chromosome as the investigated mutation. Homozygous Ovo^D /Ovo^D and heterozygous Ovo^D /ovo^+ oocytes do not develop. However after mitotic recombination oocytes, which are homozygous for the mutation and homozygous wild type for Ovo^+ develop and are fertilized.

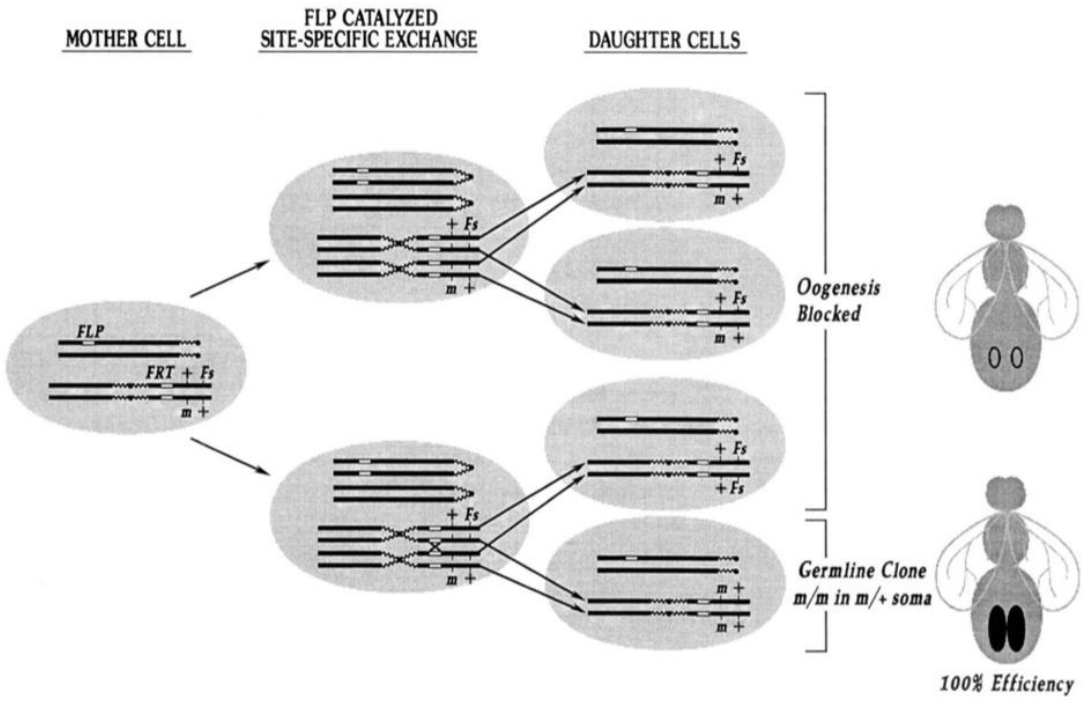


Fig. 2.5. The FLP/FRT technique to induce female germline mosaics.
FLP-recombinase induces site-specific chromosomal exchange at FRT sites. After recombination germ line clones homozygous for the mutation (a^{mut} / a^{mut}) and homozygous for the female sterile (Fs) wild type gene ($Ovo^{+)}$ are laid. In the other cells containing a wild-type copy of the mutated gene (a^{mut} /a^{+}) and a female sterile mutation (Ovo^{D}) the oogenesis is blocked (*Chou and Perrimon, 1992*).

For the induction of recombination for generation of female germ line mosaics the FLP/FRT technique is used. Compared to the induction of recombination by X-ray in earlier days, the FLP/FRT technique provides site-specific mitotic recombination with a high frequency of 90% -100% female mosaics (*Chou and Perrimon, 1992*). A transgenic line is used, which encodes the yeast recombinase FLP and another one encoding **F**LP **r**ecombinase **t**arget (FRT) sequence. The FLP recombinase is controlled by a heat-inducible promoter and thus can be activated at a particular time and promote recombination at the FRT sites. After binding to the FRT sequence the FLP recombinase subsequently catalyzes the recombination in exactly this site of the chromosome (*Golic, 1991*).

2.4 Histological Methods

2.4.1 Fixation of embryos and antibody-stainings

The flies were put in cups on top of apple juice agar plates. The plates are changed after five hours during the day and in the morning to collect eggs of different stages of development. The chorion of the eggs was removed with Sodium-Hypochlorite (NaHCl) and the embryos were fixed in 4% Formaldehyde.

- Eggs were removed of the agar plate with some water and a paint brush and put into fine wire
- the wire with the eggs was placed into a 1:1 NaHCl water solution
- after max 3 min the embryos were washed for 3 times with water to remove the NaHCl
- the embryos were transferred with a paint brush into glass vials containing the fixative-heptane mix
- Incubation on a shaker for 20 min
- Remove the fixative, the lower phase in the vial, replace with Methanol
- Shake the vial strongly, or vortex for 15 sec. until the embryos fall down into the methanol phase, then the vitellin-membrane is removed
- Remove the heptane phase
- Transfere the embryos in an eppendorf tube
- Wash 3 x with Methanol

After this step the embryos can be frozen at -20°C

- Wash 3 x for 20min with 1xPBT
- Incubation for 1h in blocking solution
- Incubate the primary antibody over night at 4°C
- Wash 3 x for 20min with 1xPBT
- Incubate the secondary antibody for 2h at RT
- Wash 3 x for 20min with 1xPBT

For fluorescent stainings the embryos are embedded in mowiol/DABCO

For DAB staining the embryos were transferred into glass vessels, during the last washing step Avidine-Biotine enhancer solution was prepared

- after the last washing step the embryos incubate in AB solution for 45 min at RT
- wash 3x 5 min with 1xPBT
- add 250µl DAB staining solution and control staining under a dissection microscope
- to stop the staining reaction wash the embryos 2-3 x with 1xPBT
- for a following AP staining the embryos are washed 2x with AP buffer
- add 250µl AP staining solution and control staining under a dissection microscope, to stop the staining reaction wash the embryos 2-3 x with 1xPBT
- after the staining embryos are washed 3x 10min with 1xPBT and then incubated in 30%, 50%, 70%, 95% and 100% ethanol (5min each) followed by an incubation in 100% Acetone for 5-10 min.
- the embryos are incubated in 1:1 Acetone/Araldite over night at 4°C
- the embryos are transferred onto a slide with acetone/araldite and incubate over night at 65°C
- embedded in 100% araldite and incubated over night at 65°C

Solutions:

Fixative 4%: 3ml Heptane; 2,6 ml 1 x PBS; 0,4 ml 37% Formaldehyd

10x PBS: 1.3M NaCl, 0.07M Na_2HPO_4, 0.03M NaH_2PO_4, ad 1L H_2O, autoclave (adjust pH to 7.4)

1xPBT: 1xPBS + 0.1% Tween 20

Blocking solution: 1xPBT + 5-10% BSA

Avidin-Biotin enhancer (AB) solution (Vectastain ABC kit from *Vector Laboratories*, Burlingame, USA) 500µl PBT + 5µl solution A mix thoroughly add 5µl solution B mix thoroughly, incubate for 30 min

DAB solution: 500µl of a 1mg/ml DAB stock (3,3- Diaminobenzidine-tetrachloride); 500 µl 1xPBS, 2 µl H_2O_2 (activation of DAB)

AP Buffer: 100mM Tris pH 9,5; 50m $MgCl_2$; 100mM NaCl; 0,1% Tween 20

AP staining solution: 1ml AP-Buffer + 4,5 l NBT + 3,5 l BCIP

Mowiol: Mix 2.4g mowiol with 6ml glycerin and 6ml H2O. Incubate for 2h at RT, add 12ml 200mM Tris pH8.5 and incubate at 50°C for 3h. Centrifuge at 4000rpm for 10min and aliquot supernatant.

Araldite (50 g): 27,175 g Durcupan component A/M; 23,705 g Durcupan component B. Shake for 1 h to mix the components well. To the mixture add: 1,75 g Durcupan component C and 1,00 g Durcupan component D. Again shake for 1h to mix thoroughly.

2.4.2 List of Antibodies used in this work

Antibody	Dilution	Source
Rabbit anti β-Gal	1:1000	Cappel
Mouse anti β-Gal	1:100	DSHB
Mouse anti CD2	1:500	Serotec
Mouse anti HA	1:1000	Roche
Mouse anti Eve	1:50-1:100	DSHB
Rabbit anti Twist	1:1000	Müller Lab

Table 2.8. Primary antibodies

Antibody	Dilution	Source
Donkey anti rabbit 488	1:200	Molecular probes
Donkey anti mouse Cy3	1:200	Jackson immuno research
Goat anti rabbit Biotin	1:200	Vector Laboratories
Goat anti mouse Biotin	1:200	Vector Laboratories
Goat anti rabbit –AP	1:800	Dianova
Goat anti mouse-AP	1:800	Dianova
DAPI	1:1000	Sigma-Aldrich

Table 2.9 Secondary antibodies

3 Eye Modifier Screen to find genetic interactors of Pbl

The Rho GEF Pbl is required for the cell shape changes during mesoderm migration and cytokinesis. During cytokinesis Pbl activates Rho1 *(Prokopenko et al., 1999)* during mesoderm migration Pbl activates Rac *(van Impel et al., 2009)*. In Pbl mutants as well as in Rac mutants the mesoderm cells do not form cellular protrusion and fail to migrate *(Schumacher et al., 2004; van Impel et al., 2009).* The upstream signals of Pbl are not known, therefore it is unclear how Pbl is regulated, localized and activated during mesoderm migration.

One experimental approach to investigate the mechanism of Pbl function in mesoderm migration is a genetic screen to find new interaction partners. The screen is based on a gain of function eye modification assay (Figure 3.1; *van Impel et al., 2009*). The protein encoded by the *UAS::Pbl*$^{DH\text{-}PH\text{-}HA}$ transgene is a constitutive active from of Pbl. The activity of many Rho GEFs is regulated by sequences in the N-terminal region. For Ect2, the mammalian orthologue of Pbl it was shown that the N-terminal BRCT domains interact with the DH and PH domains and inhibit their function *(Saito et al., 2004)*. The over-expression of *Ect2*$^{DH\text{-}PH}$ results in a constitutive activity of the protein, because this negative regulation is missing *(Schmidt and Hall, 2002; Zheng, 2001)*. Expression of the transgene *Pbl*$^{DH\text{-}PH\text{-}HA}$ in the embryo results in defects during mesoderm migration, a phenotype that is similar to the *pbl* mutant. Furthermore expression of *Pbl*$^{DH\text{-}PH\text{-}HA}$ in the eye causes severe defects in the eye morphology *(van Impel et al., 2009).*

The *Drosophila* compound eye is a good model to test interactions between different gene products in vivo. The eye is very sensitive to changes in protein amounts, for example MAPK activation by the small GTPase Ras is required for cell growth and differentiation of the photoreceptor cells. Over-expression of constitutive Ras interferes with activation of MAPK and results in eye tissue overgrowth and apoptosis *(Halfar et*

al., 2001). The over-expression of constitutive active Pbl causes similar defects suggesting that $Pbl^{DH\text{-}PH\text{-}HA}$ interacts with similar pathways in the eye.

A special expression vector is used to express $Pbl^{DH\text{-}PH\text{-}HA}$ in the eye with the UAS/Gal4 system (*Brand and Perrimon, 1993*; for details see methodical part 2.3.3). The vector pGMR (**g**lass **m**ultimer **r**eporter) contains truncated binding sites of *glass*, the transcription factor for the promoter of the *Drosophila* gene *roughened*. It is expressed in all cells of the morphogenetic furrow of the eye disc during larval development and in all retinal cells in the pupae *(Hay et al., 1994)*.

The compound eye of *Drosophila* is composed of ca. 750 hexagonal shaped ommatidia, which are arranged in a regular pattern (Fig. 3.1 A). The expression of $Pbl^{DH\text{-}PH\text{-}HA}$ with the eye specific driver *GMR::Gal4* results in a remarkable rough eye phenotype of the eye. The hexagonal shape of the ommatidia is lost and therefore the morphology of the compound eye. Some ommatidia are completely gone and replaced by cuticle (Fig. 3.1 B). The expression of $Pbl^{DH\text{-}PH\text{-}V531D}$, a transgene in which the DH-domain is mutated in such way that its exchange activity is lost, does not result in a rough eye phenotype, implicating that the defects are caused by the over-activation of Pbl substrates (Fig. 3.1 C). Moreover the expression of $Pbl^{DH\text{-}PH\text{-}HA}$ in flies, which are heterozygous for *pbl* loss of function allele, suppress the rough eye phenotype (Fig. 3.1 D). These results confirm that $Pbl^{DH\text{-}PH\text{-}HA}$ is a gain of function of Pbl.

Pbl acts through activation of down-stream GTPases and therefore the reduction of Pbl substrates Rac and Rho1 should suppress the defects in the compound eye caused by $Pbl^{DH\text{-}PH\text{-}HA}$. In fact $Pbl^{DH\text{-}PH\text{-}HA}$ over-expression in flies carrying a mutation in Rho1 lead to a suppression of the rough eye phenotype (Fig. 3.1 E). In *rac1* and *rac2* mutants a suppression of the eye phenotype can be observed as well (Fig. 3.1 F). The co-expression of *UAS::Rac1* and *UAS::Rac2* together with over-expression of $Pbl^{DH\text{-}PH\text{-}HA}$ in the eye, results in a strong enhancement of the eye defects; most of those flies die

during development. Only a few escaper flies hatch, which failed to develop any eye structures (Fig. 3.1 G). The expression of $Pbl^{DH\text{-}PH\text{-}HA}$ in mutants of other members of the Rho GTPase family in *Drosophila*, RhoL and Cdc42, did not result in modification of the rough eye phenotype. Hence the genetic interaction of $Pbl^{DH\text{-}PH\text{-}HA}$ with Rho1 and Rac is specific for these RhoGTPases.

The genetic interactions prove that $Pbl^{DH\text{-}PH\text{-}HA}$ is a gain of function allele of Pbl and that the removal of Pbl interacting proteins in the eye modifies the rough eye phenotype of the compound eye. The over-expression of constitutive active Pbl shows that $Pbl^{DH\text{-}PH\text{-}HA}$ interacts with the activation of Rac and Rho1 pathways in the eye.

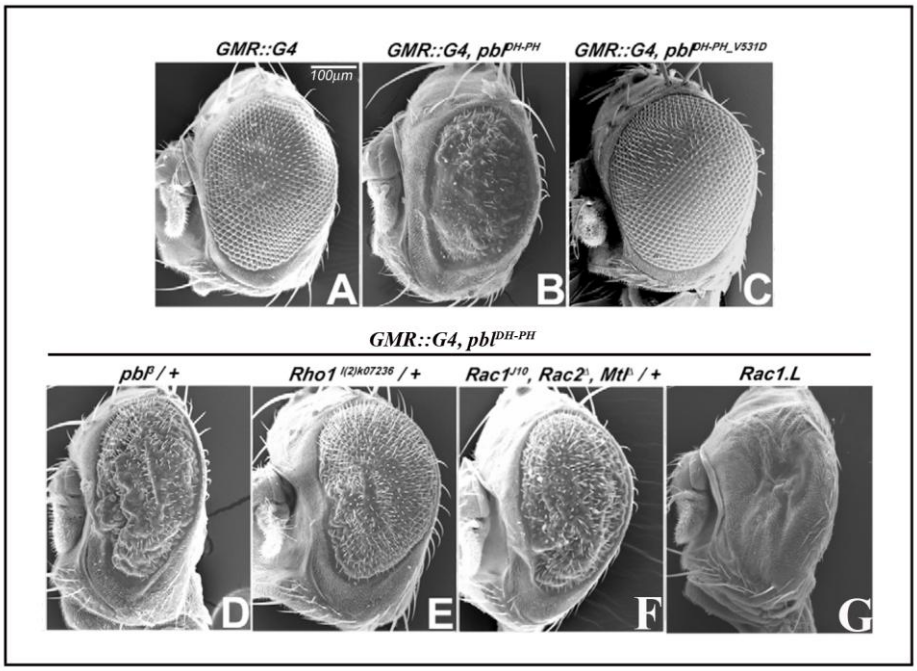


Fig.3.1 Eye modification assay.
SEM pictures of *Drosophila* compound eyes showing defects in the morphology., all pictures are to the same scale bar, 100µm. (A) The eyes of *GMR::Gal4* flies are normally developed. The ommatidia are shaped hexagonal and the bristles are distributed regularly. (B) The expression of $Pbl^{DH\text{-}PH\text{-}HA}$ with *GMR::Gal4* results in severe defects of the eye morphology, a so-called rough eye phenotype. The ommatidia loose their hexagonal shape, round up and are strongly reduced. The bristles are mis-distributed. (C) Expression of $Pbl^{DH\text{-}PH\text{-}V531D}$ does not cause a rough eye phenotype. (D) In *pbl* heterozygous mutant flies the defects after expression of $Pbl^{DH\text{-}PH\text{-}HA}$ are not as strong as in wild type. (E, F) The rough eye phenotype is suppressed in heterozygous mutants for the two Pbl substrates, *rho* and *rac*. (G) After over-expression of Rac together with the over-expression of $Pbl^{DH\text{-}PH\text{-}HA}$ the rough eye phenotype is strongly enhanced. The ommatidia and bristles are absent and instead cuticle is formed were the eye tissue should develop (*van Impel et al., 2009*).

Principle of the Screen

The gain of function eye modification assay revealed that *Pbl*$^{DH\text{-}PH\text{-}HA}$ is a gain of function allele of Pbl and that the compound eye is an appropriate model to investigate genetic interactions of *Pbl*$^{DH\text{-}PH\text{-}HA}$ with specific targets. Hence genes that genetically interact with Pbl can be detected when their function is lost. The idea of the screen was to express the constitutive active form *Pbl*$^{DH\text{-}PH\text{-}HA}$ in the eyes of flies that carry chromosomal deletions or mutations in single genes, to find potential genetic interactors of Pbl. An enhancement of the rough eye phenotype indicates that the affected gene might negatively regulate Pbl function and thus lack of this gene increases the morphological defects.

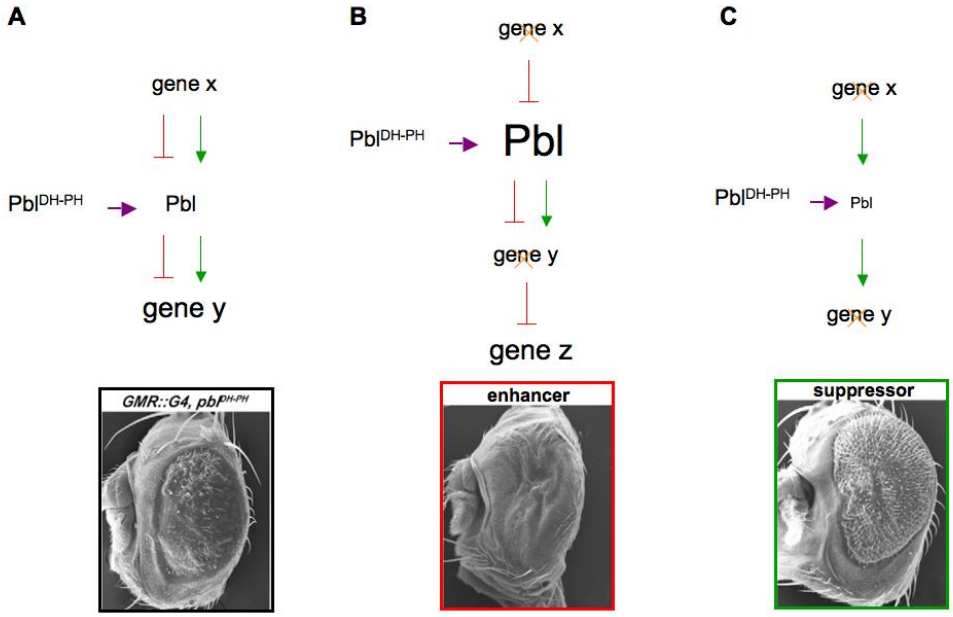


Fig. 3.2 The principle of the eye modifier screen.
(A) GMR>>Pbl $^{DH\text{-}PH}$ causes defects in the eye morphology in wild type flies, because over-expression of *Pbl*$^{DH\text{-}PH}$ (indicated by the purple arrows) increased the overall Pbl protein amount in the eye, which interferes with Rac and Rho signalling pathways by changing the expression of downstream targets. (B) The rough eye phenotype is enhanced (red), when the endogenous amount of Pbl protein and its targets is higher. Genes having an inhibitory function on Pbl (gene x) might be removed. Similarly the loss of genes downstream of Pbl (gene y) and inhibit other genes (gene z) can also enhance the rough eye phenotype. (C) Suppression (green) of the rough eye phenotype occurs when genes are lost, which positively regulate Pbl activity (gene x),the amount of endogenous Pbl protein is reduced, therefore the affect of the expression of *Pbl*$^{DH\text{-}PH}$ is less strong. Furthermore, when genes which are activated by Pbl (gene y) are missing, a suppression can be observed, because the strong over activation of downstream targets by *Pbl*^{DHPH} is reduced.

For instance, an enhancer mutation might lead to a larger amount of Pbl protein or the loss of downstream targets of Pbl, which inhibit other gene functions. This kind of mutations might lead to an increase in the amount of Pbl targets (Fig. 3.2 B). On the other hand a reduction in the amount of Pbl protein or Pbl targets leads to suppression of the rough eye phenotype. This may happen by the loss of genes activating Pbl, or being activated by Pbl (Fig. 3.2 C).

Procedure of the screen

For a constitutive expression of *UAS::Pbl*$^{DH\text{-}PH}$ with *GMR::Gal4* in the compound eye, a transgenic line was produced by meiotic recombination, *GMR>>Pbl* $^{DH\text{-}PH\text{-}HA}$. Males, which carry different deletions or mutations on the second and third chromosomes, were crossed to *GMR>>Pbl*$^{DH\text{-}PH\text{-}HA}$ females (Fig.3.3). The progeny were screened for a modification of the rough eye phenotype.

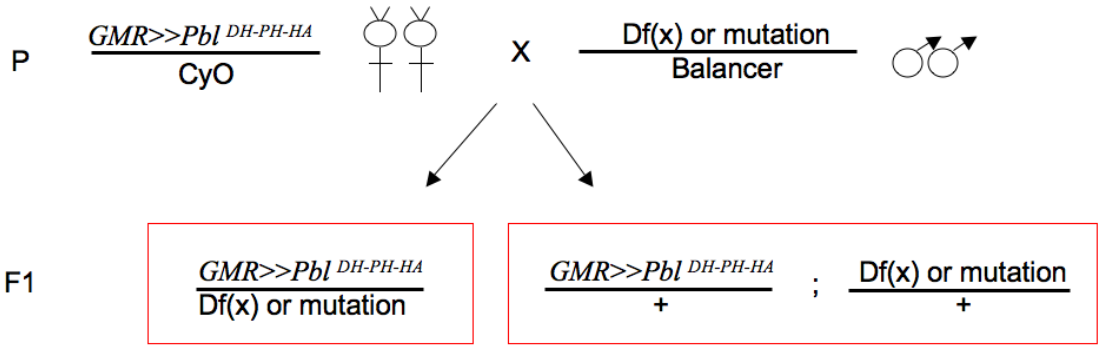


Fig.3.3 Crossing scheme of the screen.
Females expressing the *Pbl*$^{DH\text{-}PH\text{-}HA}$ transgene with *GMR::Gal4* (*GMR>>Pbl*$^{DH\text{-}PH}$) are crossed to males that carry a deletion or mutation on either the first or on the second chromosome. In the F1 the deletion/ mutation is either on the second chromosome or on the third chromosome. Flies of the F1 are screened for a modification of the rough eye phenotype (boxes).

The crosses were kept on 23°C to test for suppression and on 18°C to test for enhancement, because expression of the transgene is temperature sensitive. The flies do not survive when kept on 25°C. Suppression can be observed best on 23°C, but flies

with an enhanced rough eye phenotype die at this temperature (*Dörrenhaus and Müller, unpublished*). Therefore enhancer candidates were tested on 18°C.

Candidate modifiers were tested for a function in mesoderm development. For that reason embryos being homozygous for the deletions, or chemically induced mutations were analyzed by antibody staining for defects in mesoderm spreading.

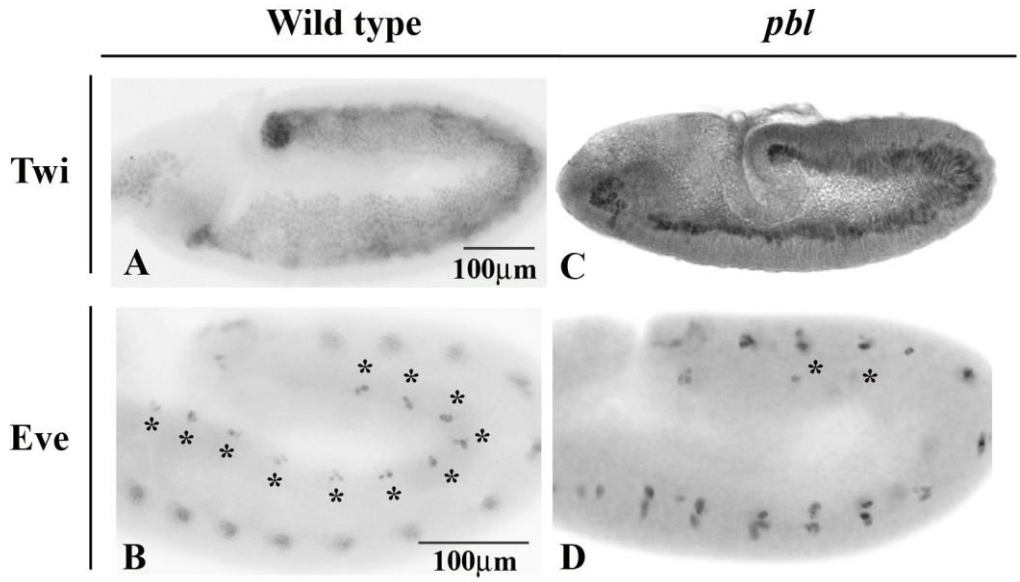


Fig. 3.4 Mesoderm migration defects in *pbl*^{3} mutants.
Wild type and *pbl*^{3} mutant embryos were stained with anti-Twi and anti-Eve antibody. Embryos are oriented anterior to the left and dorsal up. (A) The mesoderm cells migrate in a straight row in dorso-lateral direction in wild type embryos stage 9. (B) Even skipped positive cell clusters are present in all 11 hemisegments on each site of the embryo in stage 10/11 (stars). (C) Mesoderm cells migrate irregularly in *pbl*^{3} mutant embryos and do not migrate far in dorsal direction (stage 9/10 embryo, E). (D) The number of eve positive cell clusters is strongly reduced (stars).

To mark mesoderm cells antibodies against the transcription factors Twist (Twi) and Even skipped (Eve) were used. Twi is required for the development of the mesoderm and its derivatives (*Wilson and Leptin, 2000*; *Cripps and Olson, 2002*) and is expressed in the nuclei of all mesoderm cells (Fig. 3.4 A, C). Eve is expressed in the cells that give rise to a subset of dorsal muscles and pericardial cells, which are arranged in clusters of 3-5 cells in the eleven hemisegments on each site of the embryo (Fig. 3.4 B) (*Frasch et al., 1987*). Embryos stained with Twi and Eve were investigated for defects in mesoderm migration. In wild type embryos the cells migrate regularly towards the

dorsal-lateral site of the embryo (Fig. 3.4 A). The cells form a monolayer on the ectoderm and become specified to differentiate. As a result of accurate cell migration 11 Eve positive cell cluster are specified on each site of the embryo (Fig. 3.4 B). In *pbl*^{3} mutant embryos the mesoderm cells do not migrate and the embryo exhibits a reduced number of Eve positive cell clusters (Fig. 3.4 C, D).

3.1 Eye Modifier Screen using chromosomal deletions

In the initial deletion screen *Pbl*$^{DH\text{-}PH\text{-}HA}$ was expressed with *GMR::Gal4* in the eyes of 339 fly lines carrying chromosomal deletions. 22 candidates on the second and third chromosomes showed interaction with *Pbl*$^{DH\text{-}PH\text{-}HA}$ in the eye and 12 displayed defects in the development of the mesoderm (*Dörrenhaus and Müller, unpublished*).

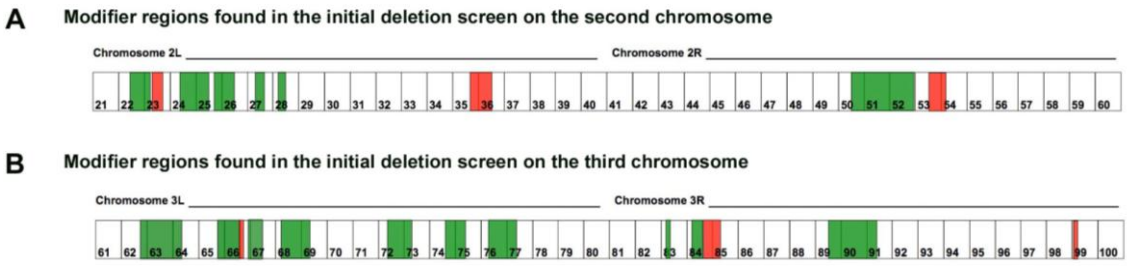


Fig.3.5 Eye modifier regions on the second and third chromosomes found in the initial screen. Regions, which showed genetic interaction with *Pbl*$^{DH\text{-}PH}$ in the compound eye are displayed in this map. Enhancer regions are indicated in red suppressor regions in green. (A) Six regions suppressing and three regions enhancing the rough eye phenotype were found on the second chromosome. (B) Ten regions suppressing and three regions enhancing the rough eye phenotype were found on the third chromosome (*Dörrenhaus and Müller, unpublished*).

The chromosomal locations of the modifier candidates found in the initial screen were defined to smaller, more accurate cytological regions, because the breakpoints of the initial deletions were not always clear. For that reason smaller deletions, which overlap with the modifier regions, were tested for rough eye modification (Fig. 3.6). Additionally embryos, which were homozygous for the smaller deletions were stained with Eve and Twi antibodies and analyzed for defects in mesoderm migration.

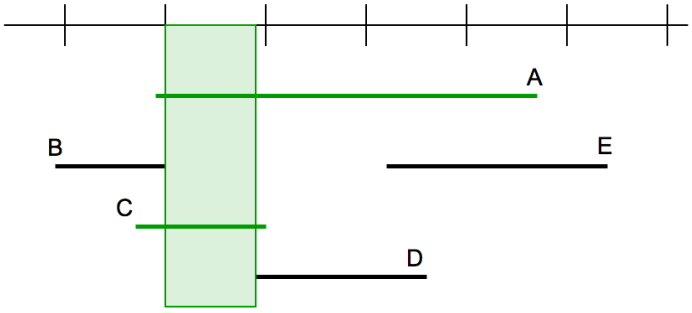


Fig. 3.6 General mapping scheme.
The extent of the chromosomal deletions is indicated by the bars. The initial deletion (A, green) shows suppression and mesoderm migration defects. Smaller deletions (B, C, D, E, black), which partially overlap with the initial deletion, were tested for rough eye modification and mesoderm migration defects. Only deletion (C, green) showed interaction. Therefore the suppressor must be in the region were deletions (A) and (C) overlap (green box).

Mapping of rough eye modifiers by analysis of mesoderm migration defects

In the initial screen twelve candidates showed modification of the rough eye phenotype and mesoderm migration defects. The mapping scheme described above was used to define the chromosomal positions of these candidates more accurately. Embryos homozygous for the smaller deletions in the modifier regions were stained with Twi and Eve. Eight regions, which showed defects in the initial screen revealed no mesoderm migration defects after fine-mapping. Possibly the mesoderm defects, observed in embryos homozygous for the larger deletions, were due to accumulative defects of many genes rather than a single locus. The other four modifiers exhibited migration defects. Mapping of theses regions is described on the following pages.

1. Suppressor region 24C3-8

The initial deletion *Df(2L)sc19-8* (Bloomington stock centre order number (Bl) #693) (Fig. 3.7) showed genetic interaction with $Pbl^{DH\text{-}PH\text{-}HA}$ and mesoderm migration defects. Closer mapping revealed that the deletions *Df(2L)ed-dp* (Bl #702) and *Df(2L)Exel6009* (Bl #7495) show defects in migration and suppression of the rough eye phenotype, with *Df(2L)Exel6009* (Bl #7495) suppression being weaker. The deletion *Df(2L)Exel8010* (Bl #7790) exhibited suppression in the initial screen but after retest

the suppression was not confirmed. Hence the suppressor was mapped to the cytological region 24C3-D4. The migration defects are displayed in the cytological region 24C3-C8 only. The genes *sloppy paired 1* and *2* (*slp1; slp2*) are localized in the cytological region 24C6-24C7. Both are transcription factors that play a role in segmentation. *slp1; slp2* double-mutants do not develop any Eve positive cell clusters in the hemisegments (*Riechmann et al., 1997*). However migration defects were never observed in *slp* mutants. It is likely that the phenotype discovered in the candidates *Df(2L)sc19-8* (Bl #693), *Df(2L)ed-dp* (Bl #702) and *Df(2L)Exel6009* (Bl #7495) is a result of deletion of the genes *slp1* and *slp2*. Whether these genes interact with $Pbl^{DH\text{-}PH\text{-}HA}$ was not examined, because the mutants were not accessible.

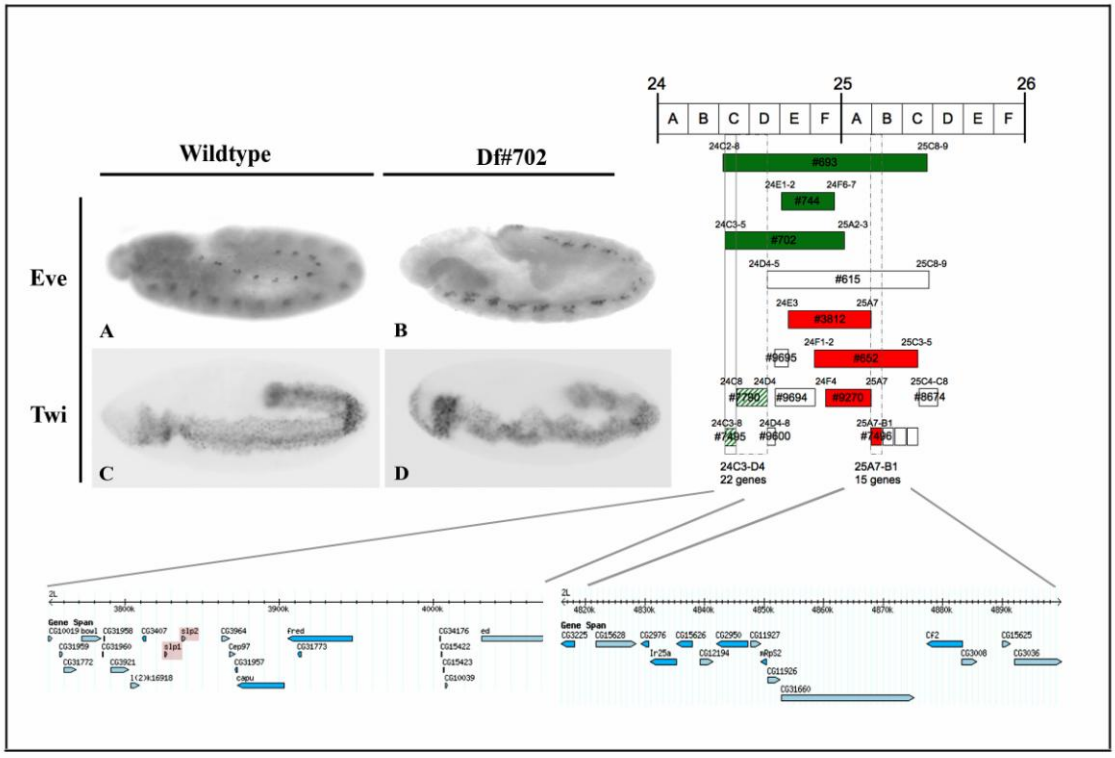


Fig. 3.7. Mapping of suppressor and enhancer in region 24C2-25C8.
The regions were mapped by testing for any interaction with $Pbl^{DH\text{-}PH\text{-}HA}$ and investigation of migration defects in the embryo. The suppression was mapped to the region 24C3-D4 containing 22 genes which are potential interactors of Pbl. Embryos are stained with anti-Eve and anti-Twi. (A, C) Wild type embryo. (B) The embryos of the deletion *Df(2L)ed-dp* (Bl #702) do not develop any eve positive cells. (D) In embryos of the deletion *Df(2L)ed-dp* (Bl #702), the mesoderm is arranged in an irregular, snake-like pattern indicating a defect in mesoderm spreading.

The deletions *Df(2L)sc19-3* (Bl #3812), *Df(2L)sc19-6* (Bl #652), *Df(2L)ED250* (Bl #9270) and *Df(2L)Exel6010* (Bl #7496) show an enhanced rough eye phenotype. The

enhancer region was mapped to the cytological region 25A7-B1 and contains 15 annotated genes. The region was not investigated further, because the modifier did not display migration defects and the deletion *Df(2L)sc19-1* (Bl #615) did not show enhancement, although it overlaps with the enhancer region 25A7-B1.

2. Enhancer region 36A12-B1

In this region the deletions *Df(2L)cact-255rv64* (Bl #2583) and *Df(2L)Exel6039* (Bl #7522) displayed enhanced modification of the rough eye phenotype (Fig. 3.8) and sporadic missing Eve positive cell clusters but never less than ten (Fig. 3.6 B). In some embryos homozygous for the deletions *Df(2L)cact-255rv64* (Bl #2583) and *Df(2L)Exel6039* (Bl #7522) the mesoderm failed to invaginate correctly (Fig. 3.6 D, E) but in the majority of embryos the development of the mesoderm continued normally. 13 annotated genes are located in this cytological region 36A12-B1.

Flies containing mutations in genes uncovered by fine-mapped deletions were tested for genetic interaction with $Pbl^{DH\text{-}PH\text{-}HA}$ in the eye. Unfortunately loss of function mutations were not available for the 13 genes located in this region. P-element insertions in the 13 genes were examined to test if the genes interact with Pbl genetically. P-elements are transposons that are randomly inserted into the genome and can be mobilized. They interfere with or even can abolish the proper function of the gene they are close to or localized in. However in most cases they do not mimic the loss of function phenotype of a gene. P-element insertions in the genes *VhaSFD, CG17996, CG17331 (Lsm7), ChlD3, CG17912* and *gluon (glu)* were tested for an interaction, but enhancement of the rough eye phenotype was not observed in any of the lines (table 2; appendix). The expression of dsRNA of the gene *CG17331,* whose function is pretty much unknown, results in defects during ventral furrow formation (*Gong et al., 2004*). There is no loss of function mutation available so far, but possibly the RNAi transgene

could be tested for genetic interaction with Pbl. Another interesting candidate in this region is the gene *glu*, which is required for mitosis. The mutation in $glu^{88\text{-}37}$/*CyO* did not show any modification of the rough eye phenotype, but the nature of the mutation is not known. A loss of function mutant exists, but is not available.

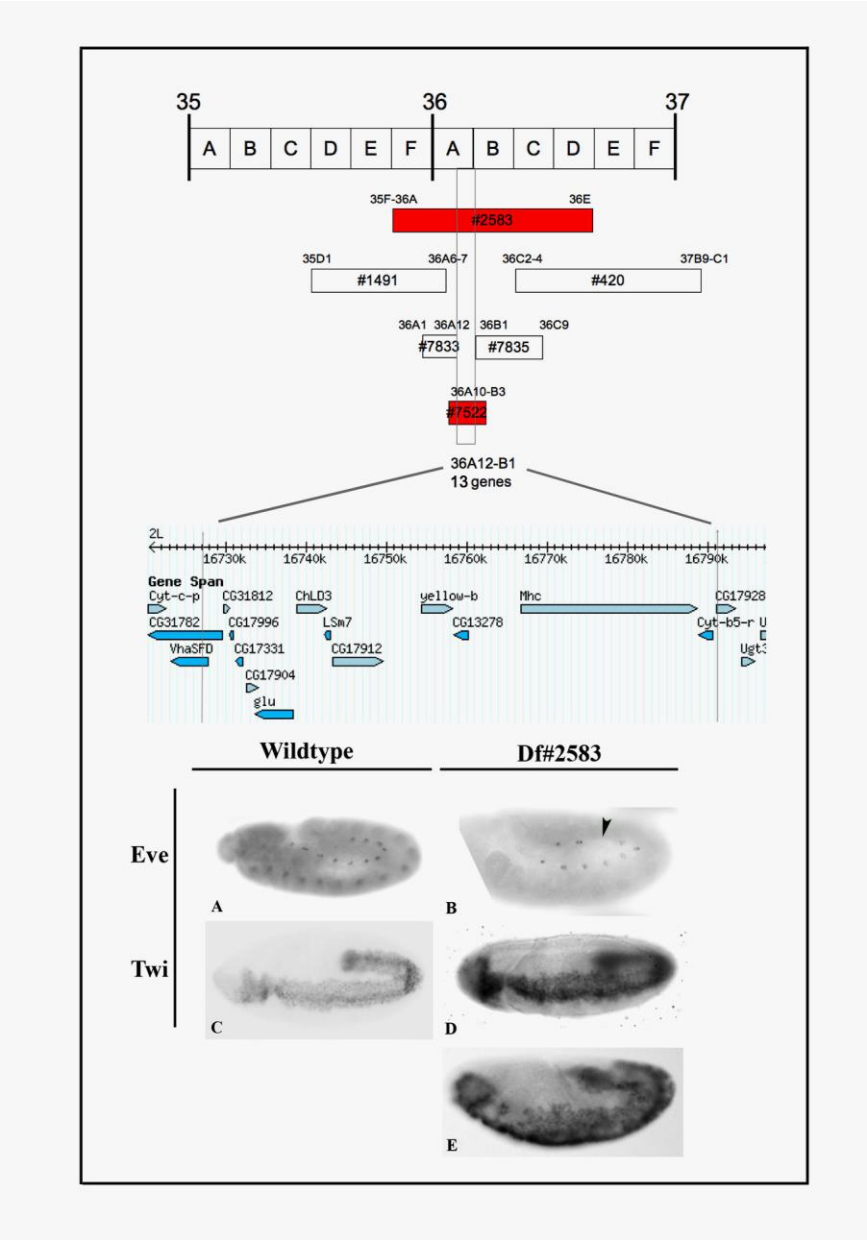


Fig. 3.8. Mapping of Enhancer region 36A12-B1.
The deletions *Df(2L)cact-255rv64 (Bl #2583)* and *Df(2L)Exel6039* (Bl #7522) show enhancement of the rough eye phenotype. The deletions *Df(2L)r10* (Bl #1491), *Df(2L)TW137* (Bl #420), *Df(2L)Exel7066* (Bl #7833) and *Df(2L)Exel8036* (Bl #7835) do not interact with $Pbl^{DH\text{-}PH\text{-}HA}$. 13 genes are mapped to the region 36A12-B1. (A, C) Wild type embryo. (B) In some embryos of deletion *Df(2L)cact-255rv64* (Bl #2583) eve cell clusters are missing (B, arrow), but most embryos show normal mesoderm cell migration, sporadic defects in invagination and migration can be observed (D, E).

3. Suppressor region 27C2-C4

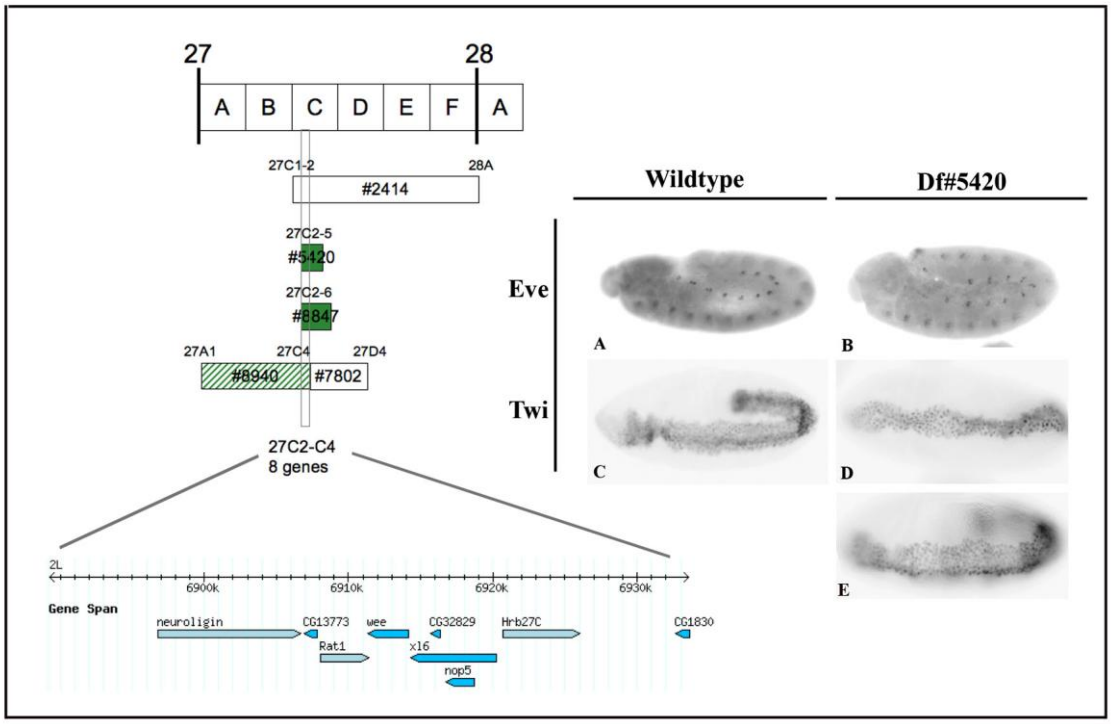


Fig. 3.9 Mapping of suppressor region 27C2-C4.
The deletions *Df(2L)Dwee1-W05* (Bl #5420) and *Df(2L)BSC108* (Bl #8847) show suppression of the rough eye phenotype. The suppression observed in deletion *Df(2L)ED6569* (Bl #8940) is weaker as in the other two. The deletion lines *Df(2L)spdƒj2ƒ* (Bl #2414) and *Df(2L)Exel7029* (Bl #7802) do not exhibit a modification of the phenotype. The migration defects that were found in the initial screen could not be confirmed. (A, C) Wild type embryo. (B) All embryos homozygous for the deletion *Df(2L)Dwee1-W05* (Bl #5420) displayed all 11 eve cell clusters. (D) Only sporadic migration defects could be observed stage 7 embryos in *Df(2L)Dwee1-W05* (Bl #5420). (E) Most of the embryos show a normal development. Eight genes are mapped to the regions where the suppressors overlap (without line *Df(2L)spdƒj2ƒ* (Bl #2414)).

The genetic suppressor effect was defined to the cytological region 27C2-C4, containing eight annotated genes. The deletions *Df(2L)Dwee1-W05* (Bl #5420), *Df(2L)BSC108* (Bl #8847) and *Df(2L)ED6569* (Bl #8940) showed suppression of the rough eye phenotype, while *Df(2L)ED6569* (Bl #8940) suppression was weak (Fig. 3.9). The deletions *Df(2L)spdƒj2ƒ* (Bl #2414) and *Df(2L)Exel7029* (Bl #7802) did not genetically interact with $Pbl^{DH\text{-}PH\text{-}HA}$ in the compound eye. The embryos homozygous for the deletions did not exhibit mesoderm migration defects (Fig. 3.9 B, E), but some of the embryos showed defects in mesoderm migration in the initial screen. All Eve positive hemisegments (Fig. 3.9 B) could be observed and the spreading of the

mesoderm cells was normal (Fig. 3.9 E). Sporadic invagination defects were noticed in a few embryos (Fig. 3.9 D).

4. Suppressor Region 72D10-F1

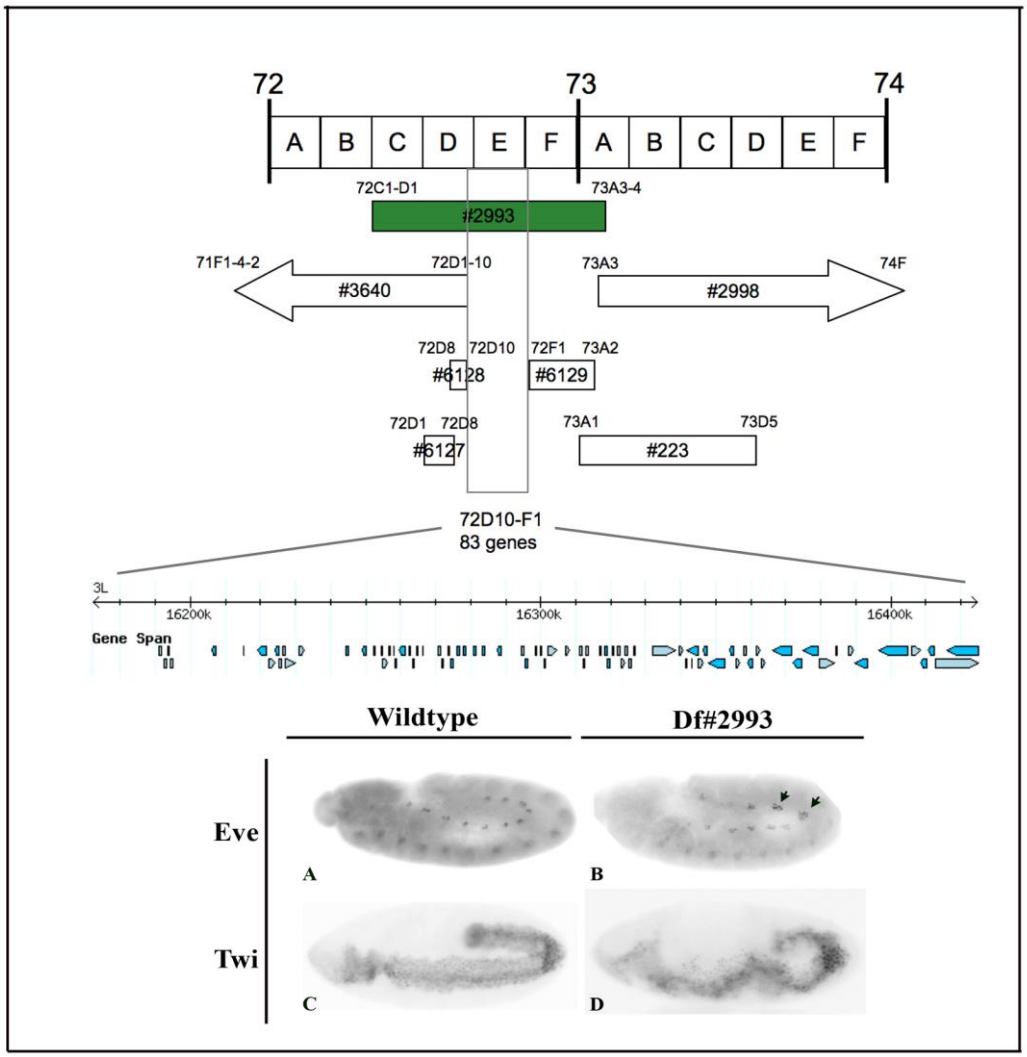


Fig. 3.10. Mapping of Suppressor region 72D10-F1.
Suppression can be observed in deletion *Df(3L)st-f13* (Bl #2993) after expression of *Pbl*$^{DH\text{-}PH\text{-}HA}$. The embryos of this line show defects in the mesoderm. (B) The Eve staining reveals more cells in some Eve positive cell clusters (B, arrows) compared to (A) wild type embryos. (D) Defects during mesoderm migration and germ band extension are noticed in embryos stained with Twi antibody. No other deletions that show suppression could be found for this region. Therefore the suppressor was mapped to the region 72D10-F1. 83 genes are mapped to this region.

The deletion *Df(3L)st-f13* (Bl #2993) showed suppression of the rough eye phenotype and homozygous embryos exhibited sporadic defects in the spreading of the mesoderm. Less Eve cell clusters but more cells in some clusters were observed (Fig. 3.10 B). The

mesoderm cells migrate irregularly and the germ-band is twisted (Fig. 3.10 D). The deletion *Df(3L)st-f13* (Bl #2993) includes the gene *thread* (*DIAP1*)(72C1-D1), which encodes an apoptosis inhibitor. In *thread* mutant embryos morphogenesis stops after germ band extension (stage 11 of embryogenesis), the cells loose their cell adhesions and round up. The stage of developmental arrest is variable, thus some embryos undergo normal invagination and cell migration until the end of germ band extension. However some embryos show defects during early stages of germ band extension and as a result mesoderm migration is disturbed *(Wang et al., 1999)*. Therefore the phenotype observed in *Df(3L)st-f13* (Bl #2993) is probably caused by a deletion of *thread*. Thread was found in a previous screen for Pbl interactor during cytokinesis, to inteact with Pbl. However Thread is not a potential interaction candidate here, because it is not localized to the cytological region, which showed genetic interaction with Pbl. Other deletions that exist in that region do not exhibit any modification of the rough eye phenotype. More, smaller deletions were not available. There are 83 genes mapped to the region 72D10-F1, too many for further investigation in the frame of this thesis.

Mapping of modifiers by analysis of the modification of the rough eye phenotype

Some of the initial modifier candidates did not show mesoderm migration defects. However they still might exert critical functions during mesoderm migration, because many genes have maternal expression, which can mask a requirement for early embryogenesis in zygotic mutants. Maternal gene products are produced by the nurse cells as mRNAs and proteins that are placed into the oocyte during oogenesis *(Gilbert, 2000)*. Consequently the embryo can undergo early steps of embryogenesis without any transcription *(Schulz et al., 1992; Edgar and Datar, 1996)*.

For that reason the mapping was continued by analysis of the modification of the rough eye phenotype, without consideration of the migration phenotype. On the following pages modifier regions are described that were mapped more accurately with smaller deletions. Furthermore single genes mapped to the defined regions were tested for interaction with *Pbl*$^{DH\text{-}PH\text{-}HA}$ in the eye.

5. Suppressor region 76D2-D3

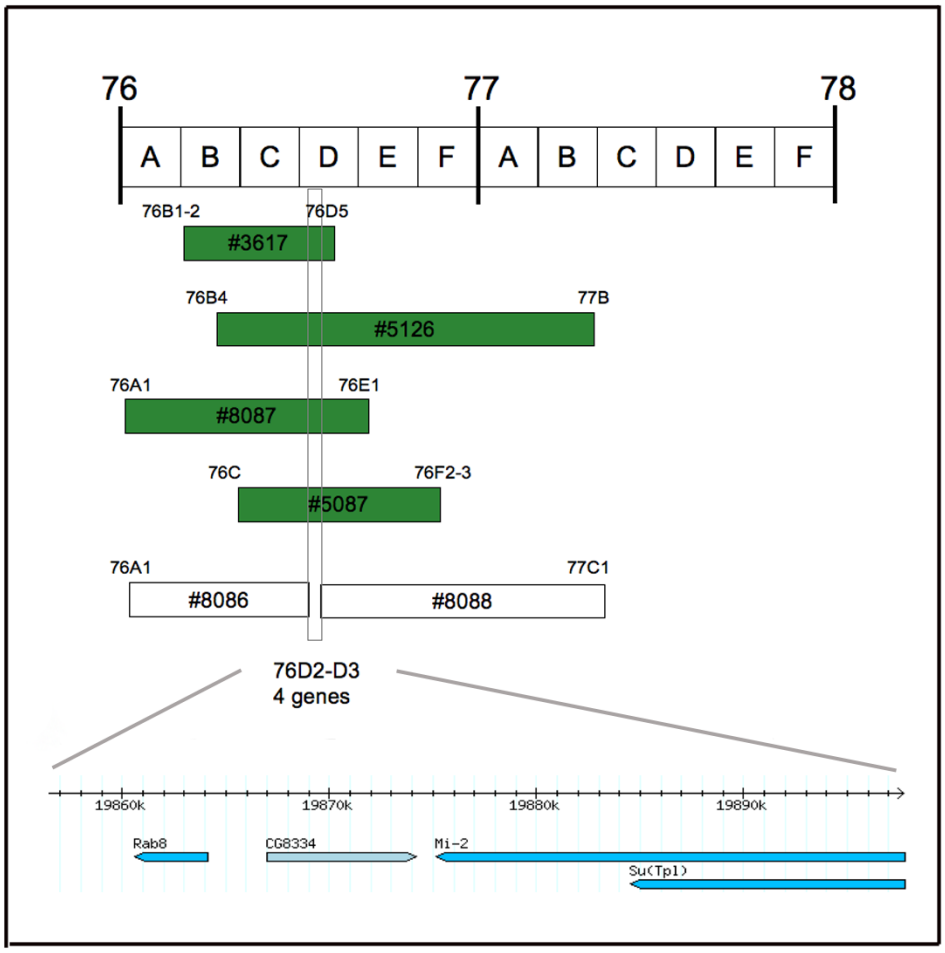


Fig. 3.11. Mapping of Suppressor region 76D2-3.
The deletions *Df(3L)kto2* (Bl #3617), *Df(3L)XS533* (Bl #5216), *Df(3L)ED229* (Bl #8087) and *Df(3L)BSC2* (Bl #5087) show suppression. The deletions *Df(3L)ED228* (Bl #8086) and *Df(3L)ED4858* (Bl #8088) do not interact with *Pbl*$^{DH\text{-}PH\text{-}HA}$. Subsequently the suppressor is mapped to region 76D2-3. Four genes are mapped to this region.

The genetic suppression effect was defined to a small region of 30Kb. The deletions *Df(3L)kto2* (Bl #3617), *Df(3L)XS533* (Bl #5216), *Df(3L)ED229* (Bl #8087) and *Df(3L)BSC2* (Bl #5087) suppressed the rough eye phenotype, while *Df(3L)ED228* (Bl

#8086) and *Df(3L)ED4858* (Bl #8088) did not (Fig. 3.11). Hence the suppressor must be located to the cytological region 76D2-D3. The genes *Rab8*, *Mi-2*, *Su (Tpl)* and *CG8334* are annotated to this region. The interaction of the genes was tested with P-element insertions in these genes, because loss of function mutations were not available for these genes at the time the screen was performed. However an interaction for any of the genes was not confirmed.

6. Enhancer region 23C5-D1

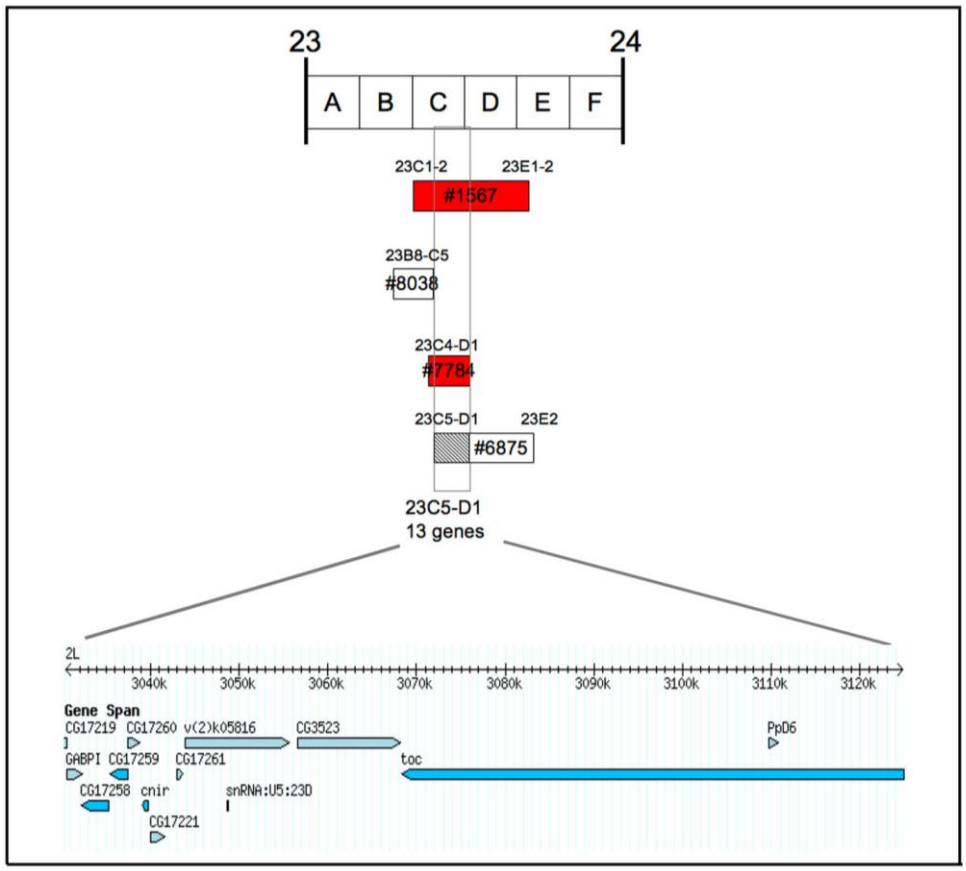


Fig. 3.12 Mapping of enhancer region 23C5-D1.
The deletions *Df(2L)JS17* (Bl #1567) and *Df(2L)Exel7014* (Bl #7784) exhibit enhancement of the rough eye phenotype. The lines *Df(2L)ED206* (Bl #8038) and *Df(2L)BSC28* (Bl #6875) do not. There might be an overlap between deletion *Df(2L)BSC28* (Bl #6875), *Df(2L)JS17* (Bl #1567) and *Df(2L)Exel7014* (Bl #7784), but the breakpoint of *Df(2L)BSC28* (Bl #6875) is not defined accurately, indicated by the grey area. 14 genes are localized in the enhancer region.

Enhancement of the rough eye phenotype was observed in the deletions *Df(2L)JS17* (Bl #1567) and *Df(2L)Exel7014* (Bl #7784). Therefore the region containing the genetic interactor of Pbl was defined to the cytological region 23C5-D1. Fourteen genes are

localized in this region. Nine genes were tested for genetic interaction with *Pbl*$^{DH\text{-}PH\text{-}HA}$ in the compound eye. A weak enhancement was observed for mutations within the gene *toucan (toc*, 23D1-D2). Toc is a microtubule-associated protein required for the stability of spindle microtubules throughout mitosis *(Mirouse et al., 2005). toc* mutant embryos could not be tested for defects in mesoderm formation, because the mutants have defects in oogenesis and are sterile (*Grammont et al., 1997*).

7. Suppressor region 51C2-C3

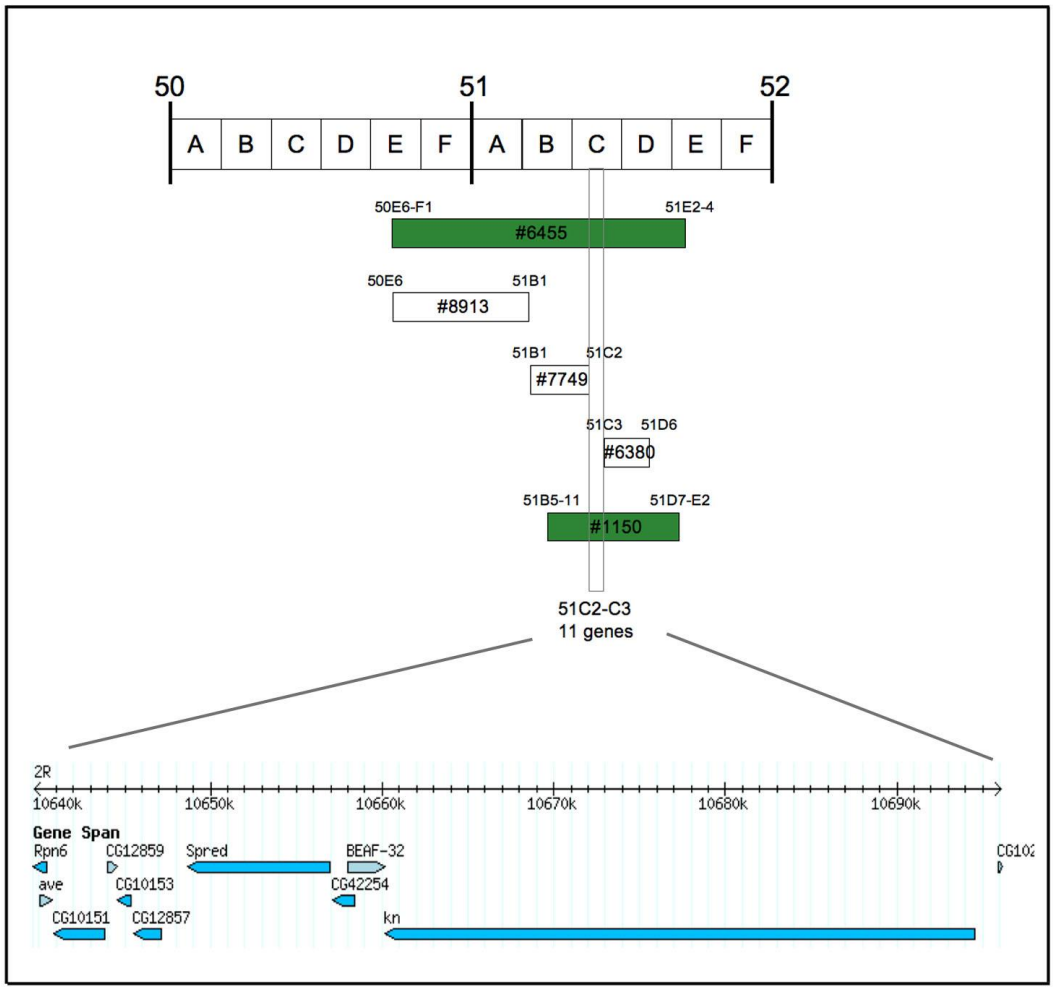


Fig. 3.13. Mapping of suppressor region 51C2-3.
The deletions *Df(2R)BSC11* (Bl #6455) and *Df(2R)knSK3* (bl #1150) showed suppression of the rough eye phenotype. The other deletions in that region, *Df(2R)ED2354* (Bl #8913), *Df(2R)Exel6284* (Bl #7749) and *Df(2R)knSA4* (Bl #6380) did not exhibit modification. So the suppressor was mapped to a region that contains 11 genes.

The genetic suppression effect was mapped to the cytological region 51C2-3, since the deletions *Df(2R)BSC11* (Bl #6455) and *Df(2R)knSK3* (Bl #1150) showed suppression of the rough eye phenotype. The region contains eleven annotated genes; six genes were tested for genetic interaction with Pbl in the compound eye using mutant alleles. No mutant lines were available for the genes *CG10153, CG12859* and *aveugle* in this region. The lines with P-element insertions in the genes *CG12857* and *BEAF-32* and a loss of function mutation in the gene *Rpn6* did not exhibit any genetic interaction in the compound eye. P-element transposon insertions in the genes *CG10151* (51C2; unknown function) and *CG42254* (51C2; chromatin insulator) slightly enhanced the rough eye phenotype, though they are in a suppressor region. One explanation would be that the P-element insertions lead to an over-expression of these genes in the eye and therefore to enhancement of the rough eye phenotype. The analysis of loss of function mutations within these genes is required, to confirm the genetic interaction with Pbl.

Mutations in the genes *spred* and *knot* were not tested because the mutations were homozygous viable strongly suggesting that the embryos develop normally. Since the screen was performed to find genetic interactors of Pbl during mesoderm migration these mutations were not suitable for the analysis of interaction.

8. Enhancer region 99A1-A6

The genetic enhancement on the third chromosome was mapped to the region 99A1-A6. The deletions *Df(3R)Dr-rv1* (Bl #669), *Df(3R)Exel6212* (Bl #7690) showed a strong enhancement of the rough eye phenotype, while the enhancement by deletion *Df(3R)ED6316* (Bl #8925) was weaker. Therefore it is possible that the enhancer region might be smaller and ranges from 99A1-A5. 18 genes localize to that region including the gene *string (stg,* 99A5). *Stg* encodes the fly homolog of Cdc25 phosphatase and is

required for cell cycle regulation. Stg interaction with Pbl was already shown in a different screen before *(Gregory et al., 2007).*

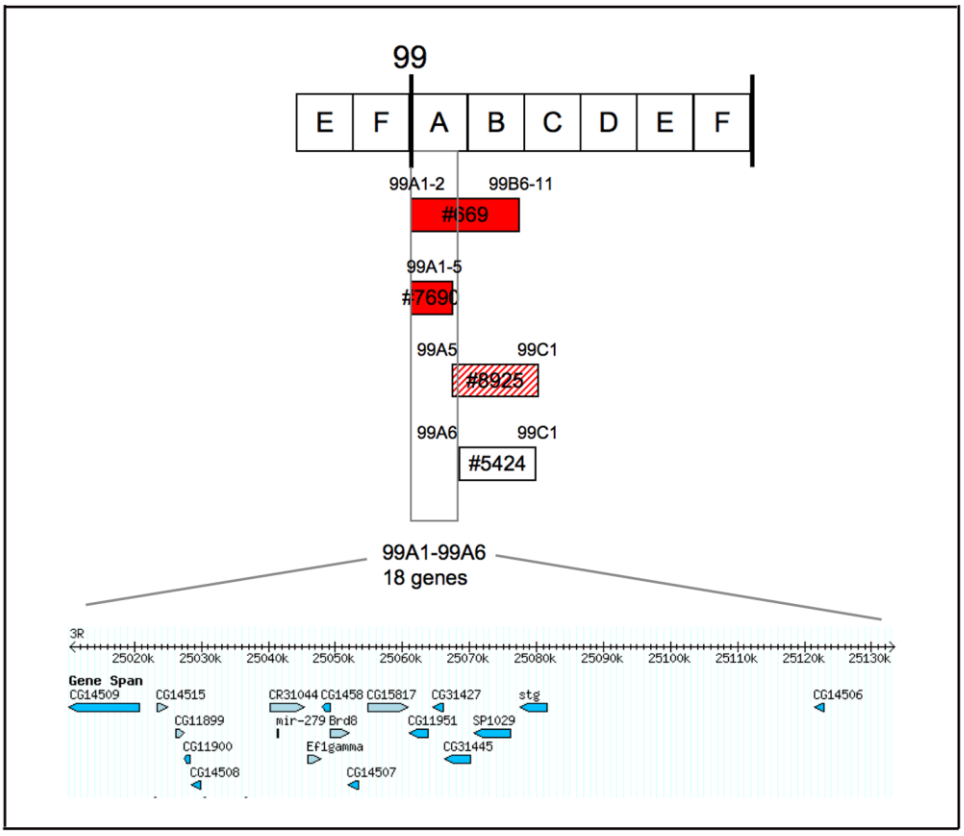


Fig. 3.14 Mapping of enhancer region 99A1-6.
The deletions *Df(3R)Dr-rv1* (Bl #669), *Df(3R)Exel6212* (Bl #7690) and *Df(3R)ED6316* (Bl #8925) enhanced the defects in the eye, with the enhancement being not so strong in line *Df(3R)ED6316* (Bl #8925). Deletion *Df(3R)01215* (Bl #5424) did not display an enhancement. In the region that was mapped 18 genes are localized.

However it might be that there is another interactor in the same enhancer region. Therefore more genes were tested for modification of the rough eye phenotype. Interaction of P-element insertions in the other genes with $Pbl^{DH\text{-}PH\text{-}HA}$ was tested in the compound eye. Interestingly the P-element insertion line $P\{Mae\text{-}UAS.6.11\}Ef1\gamma UY752^{CR31044UY752}$ of the gene *CR31044* (99A1) did exhibit enhancement. Expression of the same P-element line with *Scer\GAL47B* causes defects in the neurons of the mushroom body, in the head of the fly (*Nicolai et al., 2003*). *CR31044* is a microRNA, a small, non-coding RNA, which has putative post-transcriptional regulatory activity *(Lai et al., 2003).* P-element insertions lines of the genes *CG31445*,

CG15817, CG14507 and *Eflγ* showed weak enhancement, whereas the gene *CG11951* did not show any.

9. Enhancer region 53D11-14

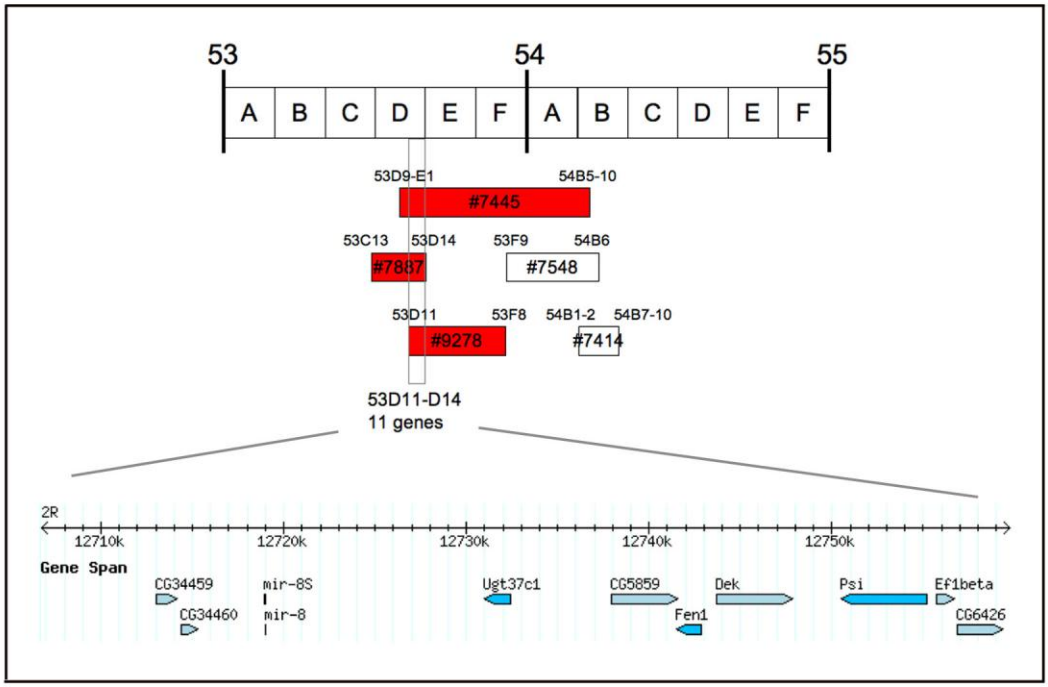


Fig. 3.15 Mapping of enhancer in region 53D11-14.
The deletions *Df(2R)BSC49* (Bl #7445), *Df(2R)Exel7145* (Bl #7887) and *Df(2r)ED2747* (Bl #9278) showed enhancement of the rough eye phenotype. The deletions *Df(2R)Exel6066* (Bl #7548) and *Df(2R)BSC44* (Bl #7414) did not exhibit an interaction. 11 genes map to this region.

To define the enhancer in region 53D11-D14 five deletions were tested for interaction with Pbl. The deletions *Df(2R)BSC49* (Bl #7445), *Df(2R)Exel7145* (Bl #7887) and *Df(2R)ED2747* (Bl #9278) displayed enhancement of the rough eye phenotype, whereas the lines *Df(2R)Exel6066* (Bl #7548) and *Df(2R)BSC44* (Bl #7414) did not. Based on these data the enhancer must be localized where the deletions *Df(2R)BSC49* (Bl #7445), *Df(2R)Exel7145* (Bl #7887) and *Df(2R)ED2747* (Bl #9278) overlap. P-element insertion lines of four of the eleven genes annotated in this region were tested for interaction. The genes *CG5859, CG6426* and *Ef1β* did not exhibit a modification of the eye structure, but the P-element insertion *P{EPgy2}DekEY07989* in the gene *Dek* (53D14) showed suppression of the rough eye phenotype. Dek is an mRNA processing protein in the

nucleus, but its function is unknown yet. The overexpression of the P-element insertion *P{EPgy2}DekEY07989* seemed to reduce the amount of Pbl protein, leading to suppression of the rough eye phenotype. Genetic interaction of Dek and $Pbl^{DH\text{-}PH}$ has to be confirmed by the analysis of a loss of function mutant of *Dek*, which is not available.

3.1.2 Conclusions of the eye modifier screen using chromosomal deletions

The modifier regions on the second and third chromosomes found in the initial screen were defined more precisely (Fig.3.16). However the low availability of loss of function mutants in the genes annotated to the modifier regions made it difficult to define single genes that interact with Pbl. The genes *toc, dek, CR31044, slp1 and 2, CG12859* and *CG42254* were found showing genetic interaction with Pbl in the compound eye.

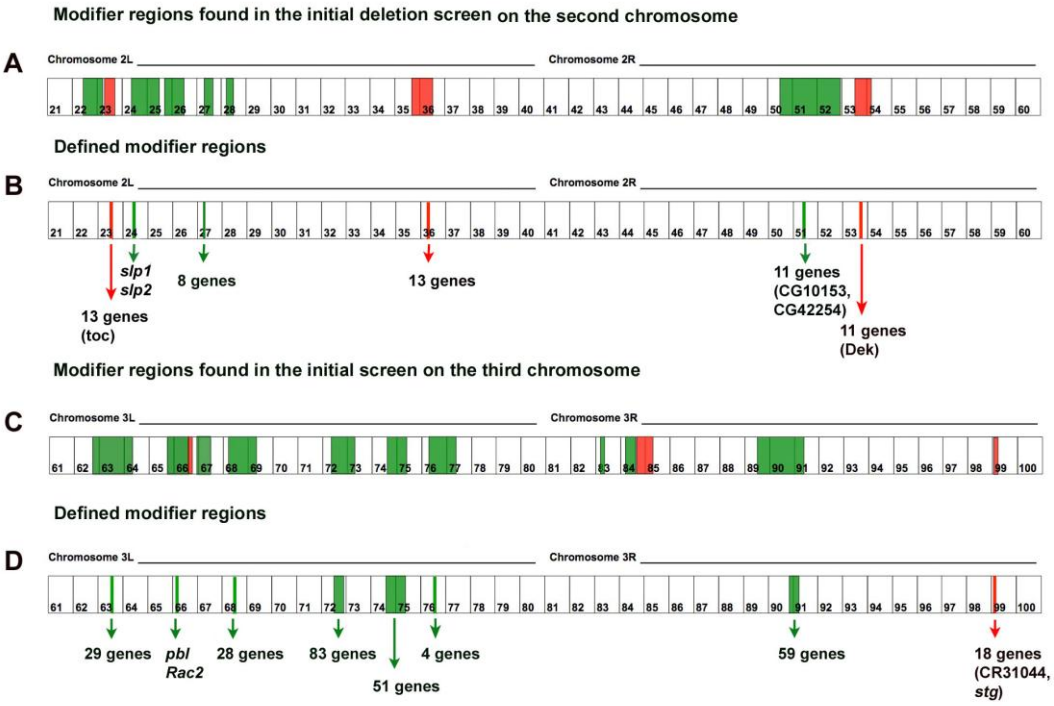


Fig. 3.16. Chromosome map of the modifier regions.
The regions containing suppressors are indicated in green and enhancers in red. (A) Six suppressor regions and three enhancer regions were found in the initial screen. (B) The regions could be mapped more accurately in the follow-up. (C) On the third chromosome ten suppressor and three enhancer regions were found initially. (D) The modifiers were defined to smaller regions.

Possible interaction of these genes with Pbl in mesoderm migration has to be investigated and is going to be deliberated more detailed on the following pages.

P-element transposon insertions in the genes *CG10151*(51C2) and *CG42254* (51C2) enhanced the rough eye phenotype caused by Pbl^{DH-PH}. *CG10151* contains a MADF domain, which specifically binds DNA and chromatin proteins. Proteins containing MADF domains are usually required for the regulation of transcription (*Maheshwari et al., 2008*). In *Drosophila* one MADF containing protein, Dorsal-interacting protein3 (Dip3) binds DNA directly via the MADF domain and stimulates synergistic activation of transcription of other genes by Dorsal and Twist (*Bhaskar and Courey, 2002*). *CG10151* could potentially be involved in the regulation of transcription of Pbl or of Pbl interacting proteins.

The gene *CG42254* encodes a chromatin insulator. Chromatin insulators control the establishment and maintenance of transcriptional domains. They can inhibit activation of transcription by blocking enhancer to interact with the promoter (*Bushey et al., 2008; Ramos et al., 2006*). Also *CG42254* might regulate the transcription of Pbl or proteins interacting with Pbl.

Another gene that is localized to the cytologic region 51C2-3 is the gene *CG10153*. Unfortunately a mutant line was not available for this gene, so interaction with Pbl^{DH-PH} could not be tested. *CG10153* is a TRAPP (Transport protein particle) component. Two TRAPP protein sub-units are known in *Drosophila* so far, *Brunelleschi (bru)* and *dBet3.* Bru is required for cleavage furrow formation during male meiosis. Bur localizes the Rab11 GTPase, which regulates membrane traffic to the cleavage furrow. In *bru* mutants cytokinesis fails during male meiosis. Pbl is required for the formation of the cleavage furrow during mitosis and meiosis as well (*Prokopenko et al., 1999; Giasanti et al., 2004*). Pbl regulates the formation of the actin-myosin contractile ring and the formation of the central spindle, which is

composed of microtuble bundles and kinesins. Afterwards the new membrane has to be formed by cleavage furrow progression. This happens by fusion of Golgi-derived vesicles, which are transported via kinesin-like protein mediated microtuble transport (*Straight and Field, 2000; Skop et al., 2001*). For that reason an interaction of Pbl in membrane traffic is possible. However no genetic interaction with Pbl and Bru during cleavage furrow formation could be demonstrated (*Robinett et al., 2009).* The function of *CG10153* is unknown yet, but its relation to the TRAPP subunit proteins make *CG10153* an interesting candidate for interaction with Pbl during cytokinesis. It might be required for the cleavage furrow formation downstream of Pbl. It would be interesting to see, if *CG10153* localizes to the cleavage furrow and/or the cell cortex. Localization at the cleavage furrow would support the model of a function of *CG10153* during cytokinesis. Additionally co-localization with Pbl at the cell cortex could indicate a function of *CG10153* during cell migration. An interesting model is the transport of Pbl or Rac in vesicles. Pbl and Rac localization was observed to be in a punctuated pattern in the cytoplasm and the cell cortex in mesodermal cells (*van Impel and Müller, unpublished*).

The gene *CR31044* (99A1) showed interaction with Pbl in the eye. *CR31044* encodes a microRNA (miRNA), which presumably exhibits post-transcriptional regulatory activity (*Lai et al., 2003*). It is required for the development of the Kenyon cell in the mushroom body (MB), the central brain of the fly. The adult MB is build from three sets of neurons that derive from neuroblasts. Primary neurites branch out of each neuron and they give rise to dendrites and axons. Axon growth involves a rapid turnover from actin polymerization to depolymerization of actin filaments (*Lee et al., 1999; Ng and Luo, 2004*). Pbl is required for the development of neurons in *Drosophila*. The proliferation of neuroblasts and axon growth are regulated by Rho1, Cdc42 and Rac. Pbl activates Rho1, which induces actin turnover during axon growth by activation

of Rock/Lim kinase (*Ng and Luo, 2004*). In another screen the gene PTP-meg was found to genetically interact with Pbl during cytokinesis. PTP-meg is involved in MB development too (*Gregory et al., 2007*). These facts lead to suggestion that Pbl potentially interacts with *CR31044* during neuronal morphogenesis. miRNAs are endogenous 22 nucleotide long RNAs, which are required for the regulation of mRNA by cleavage or interference with translation (*Bartel, 2004*). *CR31044* might regulate the expression of Pbl, or the regulation of other genes interacting with Pbl.

The gene *Dek* (53D14) showed a weak suppression of the rough eye phenotype. Dek function is unknown. However Dek is required for mRNA processing in the nucleus and it contains a SAP domain, a DNA binding motif. The human Dek is an oncogene, which is over-expressed in many human cancers and leads to increased proliferation and failure of differentiation (*Wise-Draper et al., 2009*). It is involved in many cellular processes including transcriptional repression, mRNA processing and chromatin remodeling (*Cleary et al., 2005*). Dek is another protein that possibly regulates the transcription of Pbl or proteins interacting with Pbl.

The gene *stg* (99A1-6) was mapped to an enhancer region. Stg was found in a previous genetic screen to interact with Pbl during cytokinesis (*Gregory et al., 2007*). Stg is a Cdc25 phosphatase and is involved in cell cycle progression. In *stg* mutants cell cycle arrests in G2/M transition (Edgar and O'Farrell, 1990). The blocking of mitosis in *stg* mutants does not affect mesoderm cell migration (*Schumacher et al., 2004*), therefore an interaction with Stg and Pbl during mesoderm spreading is unlikely. The rough eye phenotype is caused by interaction of $Pbl^{DH\text{-}PH}$ with both, Rho and Rac dependent processes. Therefore interaction of $Pbl^{DH\text{-}PH}$ and Stg in the eye is likely due to a suppression of the cytokinesis defects by blocking mitosis in *stg* mutants. Although $Pbl^{DH\text{-}PH}$ is able to interfere with Rho and Rac pathways in the eye, it only affects Rac dependent processes in the embryo. Over-expression of $Pbl^{DH\ PH}$ in the embryo does not

affect cytokinesis. Presumably different factors influence the interaction of Pbl with Rac and Rho in the mesoderm and in the eye. Htl is likely to be a factor why *Pbl*$^{DH-PH}$ interacts with Pbl and Rac in the embryo but not with Rho.

The gene *spred* (sprouty-related protein with EVH-1 domain) is mapped to one defined modifier region but an interaction with Pbl was not tested, because *Spread* null mutants are viable and fertile and there might be redundancy with other Spread proteins or Spry (*Gehlen and Müller, unpublished*). Spred and Spry proteins are antagonistic to Epidermal and Fibroblast growth factor signalling pathways. Spred inhibits growth factor activation of MAPK, by suppressing the phosphorylation of Raf, which is essential for activation of MAPK (*Wakioka et al., 2001*). It was shown that Spry inhibits Breathless FGF signalling during branching of the trachea in *Drosophila* (*Hacohen et al., 1998*). However a function of Spry and Spred was not revealed for Htl FGF signalling. If Pbl would interact with Spred, it would be downstream of MAPK, meaning that Pbl is probably regulated through MAPK. Spred is an inhibitor of MAPK, therefore missing of Spred would mean that MAPK is activated constitutively and Pbl is downregulated, because it suppresses the rough eye phenotype. We would need to make *spred, spry* double mutants to analyze a possible role for them in antagonizing MAPK activation.

Another interesting candidate is the gene *toc* (23D1-D2), which showed enhancement of the rough eye phenotype. Toc is a microtubule-associated protein (MAP). MAPs modulate cytoskeletal organization and dynamics in cellular processes like intracellular transport, mitosis, cell migration and differentiation. MAPs can interact with tubulin, stabilize microtubules and link them to other cytoskeletal polymers (*Maccioni and Cambiazo, 1995*). Toc protein is required for the assembling of spindle microtubules throughout mitosis (*Mirouse et al., 2005*) since Pbl is required for spindle assembly during mitosis and meiosis (*Giansanti et al., 2004*) a functional

interaction of Toc and Pbl is possible. Toc might be required for the cell shape changes during mesoderm migration downstream of Pbl and Rac. However an interaction of both during mesoderm migration is unlikely, because Toc is not expressed during mesoderm migration. Toc is required for the development of follicle cells during oogenesis, so embryos in *toc* mutants do not develop (*Grammont et al., 1997*). Furthermore Toc is highly expressed during oogenesis and only during the syncytial stages of embryonic development (*Debec et al., 2001*). Though the expression in later stages might be very weak and difficult to detect. A function of Toc in the mesoderm by expression of Toc dsRNA with *twi::Gal4* in the mesoderm should be tested to see if it affects mesoderm spreading.

Slp1 and Slp2 localize to a region showing interaction with $Pbl^{DH\text{-}PH}$.However an interaction with mutants of Slp1 and Slp2 and Pbl was not tested so far. Slp1 and Slp2 have overlapping function in segmentation and mesoderm development (*Grossniklaus et al., 1992; Cadigan et al., 1994; Riechman et al., 1997*). The *slp* genes are transcription factors, which are activated by Wg, but also on the other hand are required for the maintenance of Wg expression and therefore responsible for the heart development (*Wu et al., 1995; Jagla et al., 1997*). Slp1 and Slp2 are required for the maintenance of high Twi expression and for the development of heart and somatic mesoderm, whereas inhibiting the development of visceral mesoderm (*Riechman et al., 1997*). Nevertheless these processes are happening earlier in mesoderm development than the mesoderm spreading regulated by Htl signalling, therefore it is very unlikely that Pbl interacts with Slp1 or 2 since Pbl function was shown to be downstream of FGF signalling. Anyway a double mutant for Slp1 and Slp2 exists and should be tested for interaction with $Pbl^{DH\text{-}PH}$ in the eye.

3.2 Eye Modifier Screen using chemical mutagenesis

The modifier screen using chromosomal deletions revealed regions containing genetic interactors of Pbl. These genetic interactors can be detected by investigation of modification of rough eye phenotype in the compound eye caused by gain of function of Pbl. Unfortunately many of the genes located in the modifier regions could not be tested for genetic interaction, because of the limited availability of mutations in these genes.

Since there are many regions containing potential interactors of Pbl a second screen was performed. For that reason flies were mutated with Ethylmethane sulfonate (EMS) after a modified protocol of Grigliatti (1986) (see protocol in the methodical part 2.3.5). EMS commonly induces single base-pair exchanges, G/C to A/T, by methylation of Guanine. This frequently leads to nonsense and missense mutations, which result in loss of function mutations in single genes. These mutant flies can be tested for an interaction with $Pbl^{DH\text{-}PH}$ regarding to a modification of the rough eye phenotype. Finally the modifier genes containing EMS induced mutations can be mapped to a cytological region found in the first deletion screen.

The use of mutations in single genes to analyze genetic interaction with Pbl and to examine defects during mesoderm migration is an advantage compared to the first screen. On the other hand, EMS can induce multiple base-pair exchanges in different genes, thus there is a possibility of mutations in more than one gene on the same chromosome. The consequence is that accumulative effects of different genes could be misinterpreted as interaction with a single locus mutation, or potential genetic interactors cannot be found, because other mutations mask the interaction.

Screen summary

For the EMS modifier screen in total 8430 male flies were mutagenized with 25mM EMS. These males were crossed to female virgin flies that express the $Pbl^{DH\text{-}PH\text{-}HA}$ transgene in the eye. The flies were kept at 22°C, because compared to the initial

deletion screen many flies died at 23°C. 23821 male flies of the progeny were screened for a suppression of the rough eye phenotype. Potential suppressing candidates were selected and crossed to flies that carry balancer-chromosomes with markers to separate the mutations on the second and third chromosome. 341 suppressing mutations mapped to the second chromosome and 561 to the third. Mutations on the X chromosome were not selected. After establishing balanced stocks 86 mutants on the second and 190 on the third chromosome were either lethal or carried lethal second site hits, judged by their inability to be homozygous for the mutation. These mutants were crossed to $Pbl^{DH\text{-}PH\text{-}HA}$ flies again. After the retest 20 mutations mapped to the third chromosome and three mutations on the second chromosome showed suppression (Table 3.1).

	Number of flies
Males mutagenized	**8430**
F1 males screened	**23821**
F1 Suppressors selected	**902**
Homozygous lethal stocks 2nd Chromosome	**86**
Homozygous lethal stocks 3rd Chromosome	**190**
Positive retested mutants 2nd Chromosome	**3(6)**
Positive retested mutants 3rd Chromosome	**20(7)**
Mutants show mesoderm defects	**2**

Table 3.1 Screen Summary
Males were mutagenized and crossed to females expressing *GMR>>*$Pbl^{DH\text{-}PH}$. In the F1 generation only male flies were screened and selected for outcrossing. Balanced homozygous stocks were retested for modification of the rough eye phenotype. 23 stocks were confirmed for interaction with Pbl and in two of them embryos homozygous for the mutation exhibit defects in the mesoderm morphogenesis. The numbers in brackets indicate mutations that might suppress the rough eye phenotype, but are not confirmed yet.

Screening for EMS modifier

The expression of *Pbl*$^{DH-PH}$ in EMS induced mutants revealed 23 mutations showing suppression of the rough eye phenotype in the compound eye. Images of the compound eyes of 20 mutations on the third chromosome were taken and the suppression of the rough eye phenotype of these mutants is presented below (Fig. 3. 17).

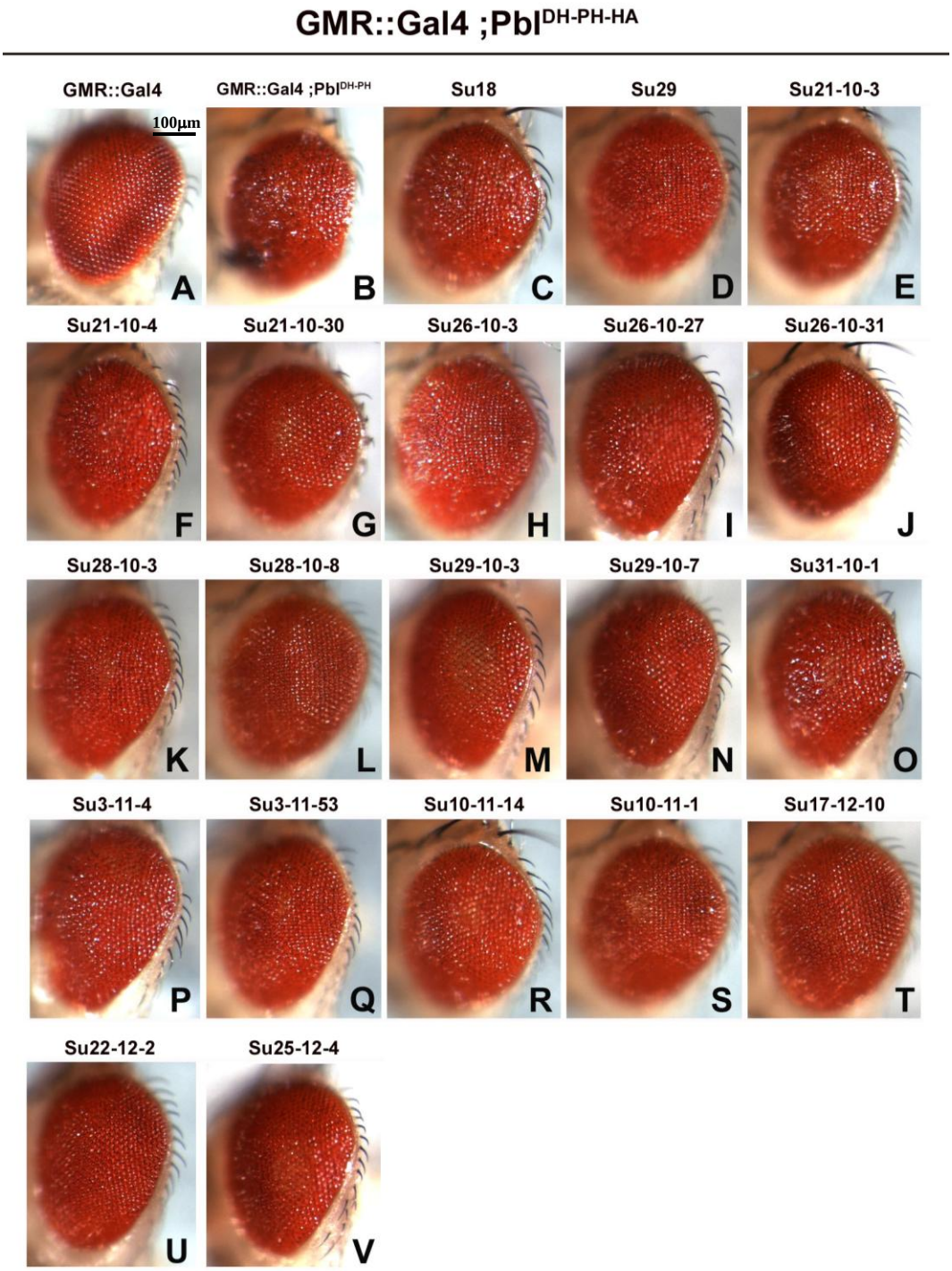


Fig. 3.17. Suppression effects in the compound eyes of EMS mutations selected in the modifier screen.

(A) The eyes of *GMR>>Gal4* flies are shaped normally. (B) Flies, which express $Pbl^{DH\text{-}PH}$ in the eye show defects of the eye morphology. The eye looks roughened and some necrotic tissue is visible. (C-V) In flies, which carry EMS mutations on the third chromosome the defects are weaker.

Furthermore embryos homozygous for the 20 EMS mutations, which genetically interact with Pbl, were stained with antibodies for Twi and Eve to investigate mesoderm migration. Of the 20 mutations analyzed, only embryos homozygous for the two mutations Su(3)29-10-3 and Su(3)31-10-1 displayed mesoderm migration defects (Fig. 3.18). In embryos of EMS mutant Su(3)29-10-3 severe defects in mesoderm migration were observed (Fig. 3.18. D-F).

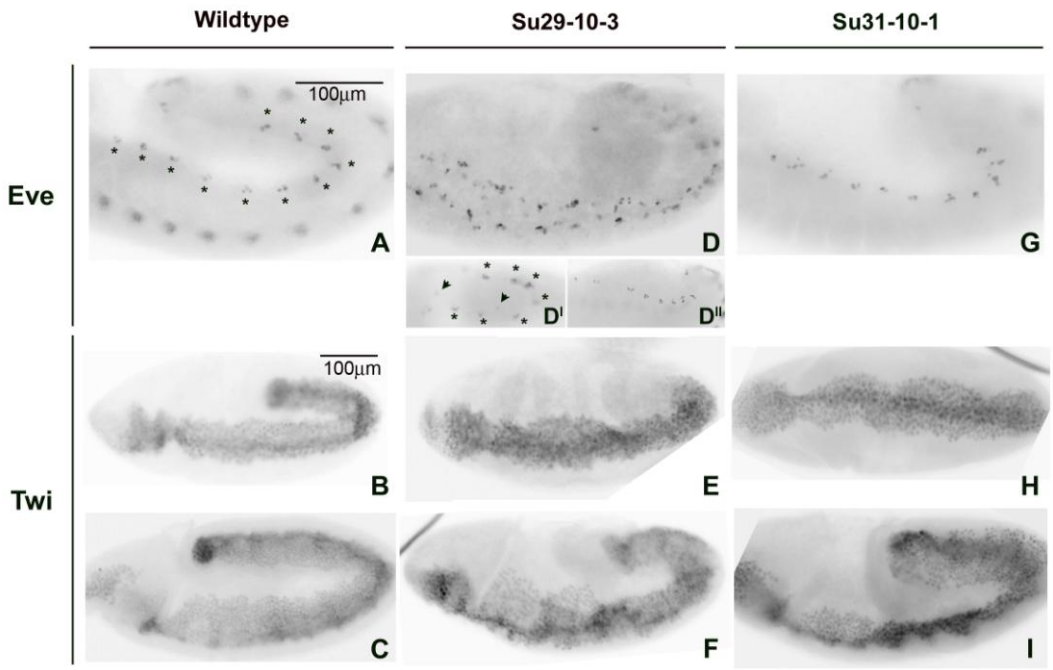


Fig. 3.18. Mesoderm migration defects in the EMS mutants Su(3)29-10-3 and Su(3)31-10-1.
The embryos are stained with antibodies against Eve and Twi. They are oriented anterior to the left and dorsal up. (A-C) Wild type embryos. (D, D^{I}, D^{II}) In embryos homozygous for the mutation Su(3)29-10-3 the number of Eve positive cell clusters is strongly reduced, but varies amongst the embryos. (D) Some embryos do not develop any Eve positive cells. (D^{I}, D^{II}) In others 7-11 cell clusters (stars) and some single cells, which are not localized in clusters can be observed (arrows). (E, F) The migration phenotype is variable. Most of the embryos show defects in mesoderm migration. (G) In homozygous mutant embryos of the suppressor line Su(3)31-10-1 all 11 Eve positive cell clusters can be observed. Cells are arranged irregular in some clusters. (H) Many embryos show irregularities in mesoderm spreading in early stages, but in later stages the mesoderm seems to develop more accurate. Only a few embryos show defects (I).

The numbers of Eve positive cell clusters are strongly reduced but vary (Fig. 3.18. D, D^{I}, D^{II}). Embryos missing all Eve cells as well as embryos, which show 22 Eve positive

cell clusters, were found (Fig. 3.18. D, $D^{I,}$ D^{II}). 82% of the homozygous mutant embryos showed missing Eve cell clusters, the other 18% exhibited a wild-type-like number of 22 Eve cell clusters.In embryos of the EMS mutant Su(3) 31-10-1 weak mesoderm migration defects were noticed. The embryos develop all Eve positive cell clusters (Fig. 3.18 G). Sporadic irregularities in mesoderm migration were observed (Fig.3.18 H) however in most embryos the mesoderm developed normally. In addition to the suppressors presented here, more suppressor mutations on the second and third chromosome were identified but need to be confirmed (indicated by the numbers in brackets Table 3.1).

The analysis of mutations on the second chromosome has to be repeated. Mutations on the second chromosome were balanced over a chromosome containing the *CyO* marker. *CyO* is a dominant mutation that results in curled wings. After crossing *mut/CyO* to *GMR>>Pbl*$^{DH\text{-}PH\text{-}HA}$*/CyO* the eyes of the flies with the genotype *mut/ GMR>>Pbl*$^{DH\text{-}PH\text{-}HA}$ were investigated for modification of the rough eye phenotype, this means only flies, which did not show curly wings were considered for eye modification analysis. However the curled wing phenotype manifests in all flies at 25°C, but at lower temperatures it is variable. Since the flies were kept on 22°C the curly wing phenotype was not displayed strongly in all flies. Therefore it was difficult to distinguish between flies that carry the mutation/*Pbl*$^{DH\text{-}PH\text{-}HA}$ and flies that are *Pbl*$^{DH\text{-}PH\text{-}HA}$*/CyO*. Additionally the *CyO* balancer chromosome suppressed the rough eye phenotype, which is another factor, that makes selection of modifiers on the second chromosome difficult. Another marker or another *CyO* balancer chromosome are going to be used for this examination.

Generation of germline-mosaics

The EMS modifier screen revealed two mutants showing genetic interaction with *Pbl*$^{DH\text{-}PH\text{-}HA}$ and mesoderm migration defects. Other EMS mutations, which genetically interact with Pbl did not display mesoderm migration defects. However

from these results it cannot be ruled out that the genes affected by these mutations might function during mesoderm spreading. The mutated genes possibly have maternal contribution and therefore did not show any defects. To investigate maternal genes it is essential to remove the maternal gene product, but this is difficult, because of the fact that the females homozygous for the EMS mutation are not viable. For that reason genetic mosaics in the female germ line were produced; in a germline mosaic fly the germ cells, which are homozygous for the mutation are created in an otherwise heterozygous animal. The generation of germ-line mosaics is a commonly used tool in Drosophila genetics and is described in the methodical part 2.3.6 after Chou and Perrimon, 1993.

The male flies, which were mutagenized with EMS in the screen, carry FRT sites on the second and the third chromosome. Therefore the 20 confirmed EMS suppressors on the third chromosome can be crossed to transgenic females carrying FLP recombinase. The recombinant females laid eggs, which were lacking the maternal geneproduct. These eggs were collected and stained with antibodies for Twi and Eve to investigate mesoderm migration. Nevertheless some recombinant EMS mutant females did not lay any eggs. This could mean that the mutated gene is needed for oogenesis. However it is very unlikely that recombinants for both chromosome arms did not lay any eggs. For instance female recombinants with the mutation Su(3)29-10-3 did not lay any eggs, meaning that genes on both arms of the chromosome were mutated. By recombination both chromosome arms were separated and mutations on both chromosome arms revealed. These mutations could have influenced each other and the mesoderm phenotype. Only one mutation showed suppression of the rough eye phenotype, it is on the left arm of the third chromosome. The embryos carrying this mutation are going to be investigated for mesoderm migration defects.

Germline clones of the other suppressor mutations were stained with Eve and Twi and were analyzed for defects during mesoderm development (see table 3 in the appendix). Some female recombinants laid only a very small number of eggs, so not all lines could be tested for defects in mesoderm development.

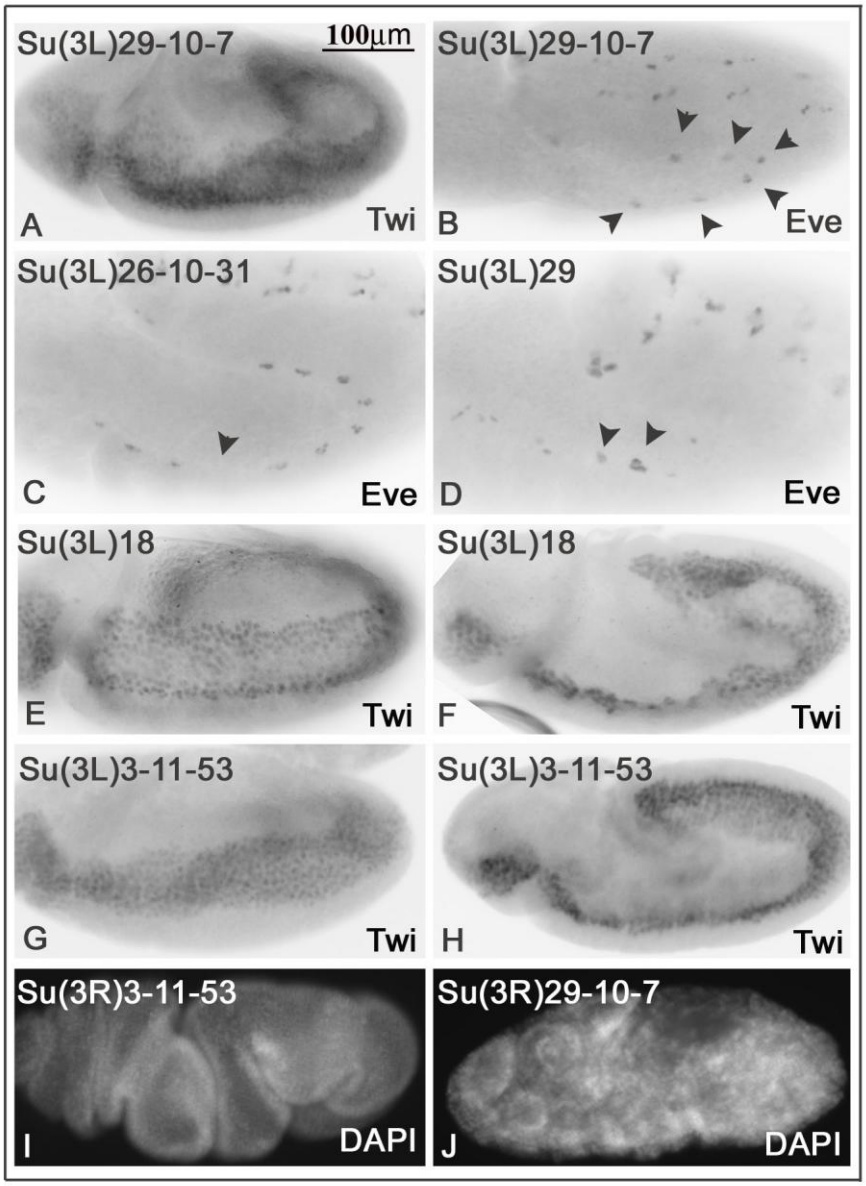


Fig. 3.19 Germline clones of the Pbl suppressor mutants showing defects in mesoderm development.
Embryos carrying the EMS induced mutations and are lacking the maternal geneproduct of the mutant gene were collected and stained with antibodies for Twi, Eve and DAPI.
(A, B, J) Embryos of suppressor mutant Su(3L)29-10-7 revealed defects in mesoderm migration and missing Eve cell clusters. A second mutation on the other arm Su(3R)29-10-7 lead to defects during embryonic development, the embryos revealed severe defcts (J). (C) Embryos of Su(3L)26-10-31 showed missing Eve cell cluster (arrow). (D) Embryos of suppressor mutant Su(3L)29 showed missing Eve cell cluster. (E, F) Embryos of line Su(3L)18 displayed sporadic defects in mesoderm spreading, in some embryos the mesoderm migrates normally (E) in other irregularities in mesoderm spreading were observed (F). (G-I) In Embryos of suppressor mutant line Su(3)3-11-53 mutations on both chromosome arms caused defects in embryogenesis. (G, H) The mutation on the left arm Su(3L)3-11-53 displayed

defects in mesoderm spreading, whereas the mutation on the right arm Su(3R)3-11-53 showed strong defects in invagination (I).

However five mutant lines displayed defects in embryonic development. Line Su(3)29-10-7 revealed mutations on both chromosome arms. Embryos of Su(3L)29-10-7 with recombination of chromosome arm 3L, showed irregularities during mesoderm migration in the Twi staining and missing Eve cell clusters (Fig. 3.19 A, B). Furthermore the same line showed a mutation on the other arm of the chromosome 3R, which leads to severe defects in embryogenesis (Fig. 3.19 J). Similar results were observed for line Su(3)3-11-53, a mutation of the left arm (3L) resulted in defects during mesoderm spreading (Fig. 3.19 G, H) but all 22 Eve cell clusters developed. Another mutation on the right arm (3R) lead to severe defects during invagination (Fig. 3.19 I).

Embryos with the mutation Su(3L)26-10-31 and mutantion Su(3L)29 revealed defects in mesoderm migration, missing Eve cell clusters were observed (Fig. 3.19 C, D). Embryos of mutant line Su(3L)18 showed sporadic defects in the spreading of mesoderm cells (Fig 3.19 F), however some embryos looked quite normal (Fig 3.19 E).

3.2.2 Conclusions of the eye modifier screen using chemical mutagenesis

The EMS eye modifier screen revealed 23 mutations, which interact genetically with Pbl in the eye. Two zygotic and five maternal gene mutations where found and are required for mesoderm development. These genes are going to be mapped and characterized and hopefully will give more insight into the function of Pbl in mesoderm cell migration through FGF signalling.

4 Functional Analysis of the PH domain and the C-terminal tail of Pbl

The underlying mechanisms in which Pbl is involved during Htl dependent mesoderm cell migration are largely unknown; in particular how Pbl is activated and localized in this process is not well understood. It is generally believed that the subcellular localization of RhoGEFs is important for their function in activating Rho GTPases. In order to understand its function it is important to investigate the localization of Pbl. During cytokinesis Pbl is localized at the cleavage furrow where it activates RhoA, which is required to assemble the contractile actin-myosin ring *(Porkopenko et al., 1999).* In many processes Rac is localized and activated at the membrane of cells and induces the formation of lamellipodia and membrane ruffles. Hence activation of Rac by Pbl possibly occurs at the cell cortex of migrating cells. During cell migration Pbl is highly enriched in the nucleus of interphase cells while low amounts of protein are present in the cytoplasm, the cell cortex and in cellular protrusions of migrating cells (Fig. 4. 1 C, D this work; *van Impel et al., 2009).*

Previous structure function analyses revealed important roles for the PH domain and the C-terminal tail for the function and localization of Pbl. The trangene $Pbl^{DH\text{-}PH}$ is expressed in the cytoplasm and localizes to the cell cortex, whereas the DH domain alone is enriched in the cytoplasm only, suggesting that the PH domain is required for cortical localization. The transgene $Pbl^{\Delta N\text{-}term}$, which differs from $Pbl^{DH\text{-}PH}$ in the presence of the C-terminal tail, localizes to the cytoplasm and the cell cortex with a stronger restriction to the cell cortex than $Pbl^{DH\text{-}PH}$. Additionally both the C-terminal tail and the PH domain alone are able to localize cortically. Suggesting both domains are important and sufficient for the localization of Pbl at the cell cortex *(van Impel et al., 2009).*

The cortical localization of the C-terminal tail and the PH domain was investigated with constructs that lack the NLS domain, so they were highly enriched in the cytoplasm and did not localize to the nucleus. To examine how the C-terminal tail and the PH domain function in the context of the full-length Pbl protein, which is not constitutively active, deletion constructs for the PH domain and the C-terminal tail were generated (Fig. 4.1 A, B, C).

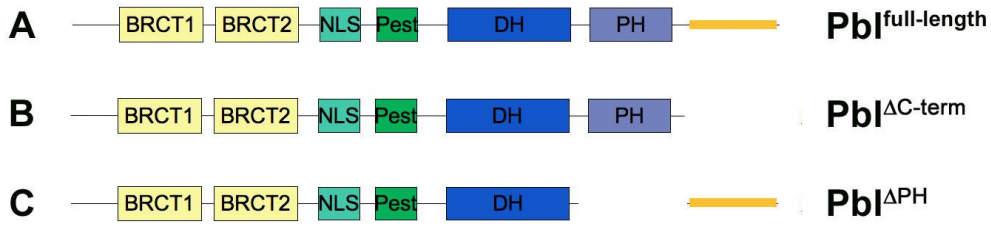


Fig. 4.1 Domain structure of Pbl, *Pbl$^{\Delta C\text{-}term}$* and *Pbl$^{\Delta PH}$*.
(A) Structure of *Pbl$^{full\text{-}length}$* which contains the entire reading frame of the PblA Isoform (transgene made by A. Van Impel). (B) Based on the PblA Isoform the construct lacking only the C-terminal tail and (C) a construct missing the PH-domain were generated and established as transgenes in flies.

4.1 The C-terminal tail and the PH domain are required for cortical localization

The transgenes, *UAS::Pbl$^{full\text{-}lengh\text{-}HA}$*, *UAS::Pbl$^{\Delta C\text{-}term\text{-}HA}$* and *UAS::Pbl$^{\Delta PH\text{-}HA}$* were expressed with *twi::Gal4* in the mesoderm (Fig. 4.2 A, B). *Pbl$^{full\text{-}lengh\text{-}HA}$* accumulates highly in the nucleus and localizes to the cytoplasm and the cell cortex (Fig. 4.2 C, D). *Pbl$^{\Delta C\text{-}term\text{-}HA}$* and *Pbl$^{\Delta PH\text{-}HA}$* are highly enriched in the nucleus (Fig. 4.2 E, F, G, H). Small amounts of both proteins were also observed in the cytoplasm (Fig. 4.2 E, F, G, H). *Pbl$^{\Delta C\text{-}term\text{-}HA}$* localizes weakly to the cell cortex in a punctuated pattern (Fig. 4.2 E, F), whereas *Pbl$^{\Delta PH\text{-}HA}$* is not found at the cell cortex at all (Fig. 4.2 G, H).

These results showed that the PH domain is essential for cortical localization of Pbl in mesoderm cells. Pbl protein lacking the PH domain was not localized to the cell cortex and the PH domain was sufficient to localize a small amount of protein to the cell

cortex when the C-terminal tail was absent. However since the cortical localization of Pbl was less pronounced when the C-terminal tail is absent, a function for the C-terminal tail in cortical localization and in stabilization of Pbl protein at the cortex has to be considered.

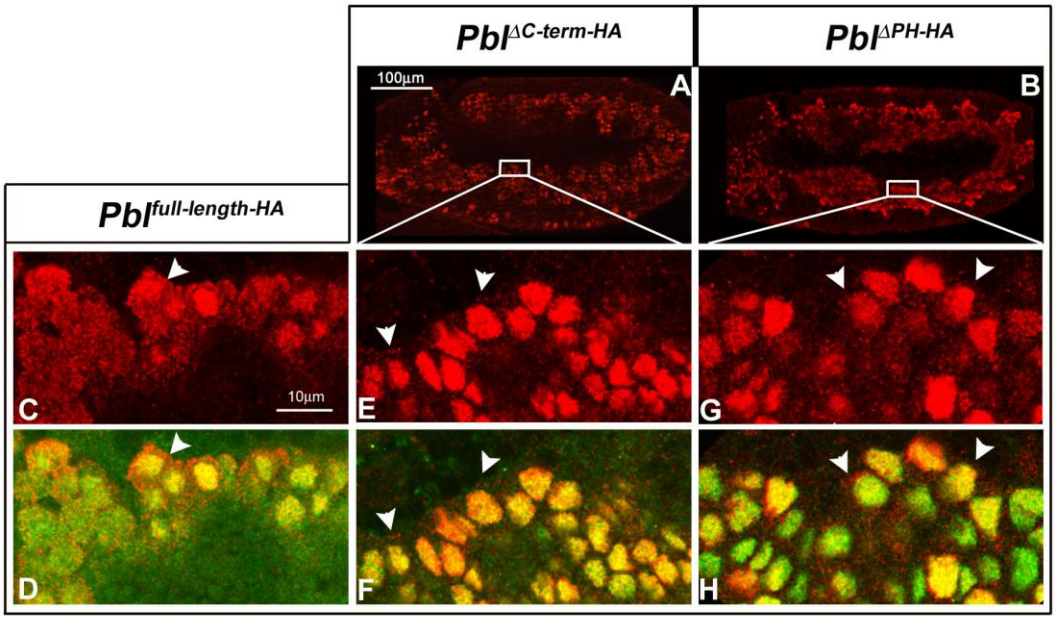


Fig. 4.2 Localization of *Pbl*$^{full\text{-}length}$ *Pbl*$^{\Delta C\text{-}term}$ and *Pbl*$^{\Delta PH}$ in mesoderm cells.
Embryos expressing *Pbl*$^{full\text{-}length\text{-}HA}$, *Pbl*$^{\Delta C\text{-}term\text{-}HA}$ and *Pbl*$^{\Delta PH\text{-}HA}$ using *twi::Gal4* in mesoderm cells are stained with anti-Twi (green) and anti-HA (red). (A, B) Expression of *Pbl*$^{\Delta C\text{-}term\text{-}HA}$ and *Pbl*$^{\Delta PH\text{-}HA}$ in the mesoderm. (C-H) Magnification of the leading edge of mesoderm cells. (C, D) *Pbl*$^{full\text{-}length\text{-}HA}$ accumulates in the nucleus and it localizes to the cytoplasm and the cell cortex (arrow C, D). (E, F) *Pbl*$^{\Delta C\text{-}term\text{-}HA}$ is enriched in the nuclei of migrating mesoderm cells, a low amount localizes to the cytoplasm and to the cell cortex in a punctuated manner (arrows E, F). (G, H) *Pbl*$^{\Delta PH\text{-}HA}$ is expressed in the nucleus and small amounts are localized in the cytoplasm (arrows G, H).

4.2 The C-terminal tail and the PH domain are important for the function of Pbl during mesoderm migration and cytokinesis

To investigate whether the PH domain and the C-terminal tail are important for Pbl function during mesoderm migration, *Pbl*$^{\Delta C\text{-}term\text{-}HA}$ and *Pbl*$^{\Delta PH\text{-}HA}$ were expressed in *pbl*^{3} mutant embryos to investigate if the proteins are able to rescue the *pbl*^{3} mutant phenotype. Previous rescue experiments with the constitutive active form of Pbl, *Pbl* $^{DH\text{-}PH}$ suggested that the PH domain plays an important role for the function of the catalytic

DH domain of Pbl. Expression of Pbl^{DH} was not able to rescue the migration defects in pbl^3 mutant embryos whereas Pbl^{DH-PH} did (*van Impel et al., 2009)*. In addition the rescue ability of Pbl^{DH-PH}, which is missing the C-terminal tail and the fact that $Pbl^{\Delta N\text{-}term}$ is not able to rescue migration, indicate an important role of the C-terminal tail for Pbl in mesoderm migration.

To investigate if the transgenes can rescue mesoderm migration defects, $Pbl^{\Delta C\text{-}term\text{-}HA}$ and $Pbl^{\Delta PH\text{-}HA}$ were expressed in pbl^3 mutant embryos. The number of Eve positive hemisegments was measured and the mean indicates if migration was rescued. In wild type embryos 22 Eve positive cell clusters are arranged. In pbl^3 mutants only one-two Eve cell clusters are defined, because mesoderm migration is disturbed. The $Pbl^{full\text{-}length}$ transgene is able to rescue the migration defects, so that 18,6 cell clusters form. The number of Eve positive cell clusters in pbl^3 mutants after expression of $Pbl^{\Delta C\text{-}term}$ ranged from 1-13, with a mean of 5.3, which represents a significant increase compared to the average of 1.7 Eve cell clusters in pbl^3 mutants (Table 4.1; Fig. 4.3 B, C). In contrast the construct lacking the PH domain, $Pbl^{\Delta PH}$ is not able to rescue migration at all; the amount of Eve positive cell clusters is similar to the amount in pbl^3 mutant embryos (Table 4.1; Fig. 4.3 B, D).

genotype	Eve positive hemisegments	S.D.	n
pbl^3/pbl^3	1,7	1,7	128
$Pbl^{full\text{-}length\text{-}HA}$; pbl^3/pbl^3	18,6	1,7	98
$Pbl^{\Delta C\text{-}term\text{-}HA}$; pbl^3/pbl^3	5,3	1,0	196
$Pbl^{\Delta PH\text{-}HA}$; pbl^3/pbl^3	2,12	0,8	198

Table 4.1 Average numbers of Eve positive cell clusters in pbl^3 mutant background after expression of $Pbl^{\Delta C\text{-}term}$ and $Pbl^{\Delta PH}$.
The table shows mean values of Eve positive hemisegments, the standar deviation (S.D.) and the number of embryos (n) analyzed.

These results revealed important roles for the PH domain and the C-terminal tail for the function of Pbl in mesoderm migration. The PH domain is indispensable for the

function of Pbl, since a protein lacking this domain could not suppress the mesoderm migration defects in *pbl*^{3} mutant embryos. The C-terminal tail is required for mesoderm migration too. However the protein lacking the C-terminal tail can still suppress the defects in *pbl*^{3} mutants significantly, suggesting that it is required for the full function of the protein, but that Pbl can also function to some extent in the absence of the C-terminal tail.

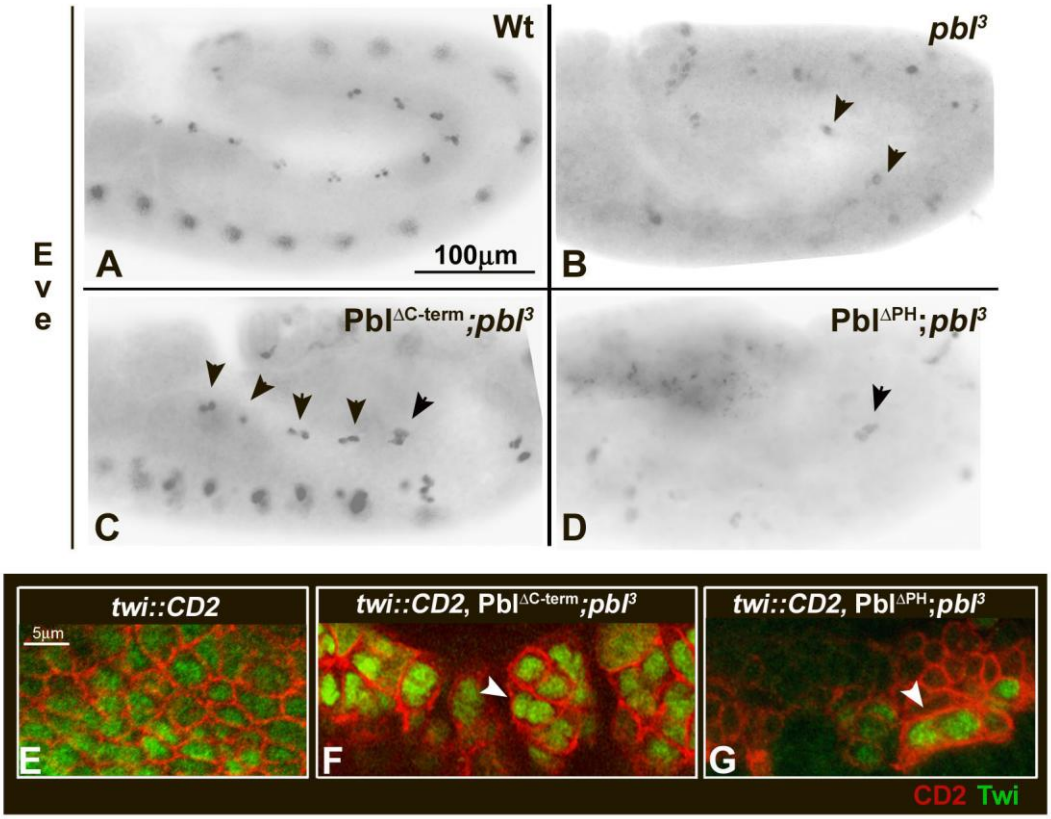


Fig. 4.3 Rescue ability of *Pbl*$^{\Delta C\text{-}term}$ and *Pbl*$^{\Delta PH}$ in *pbl*^{3} mutant embryos.
(A-D) Embryos expressing *Pbl*$^{\Delta C\text{-}term}$ and *Pbl*$^{\Delta PH}$ with *twi::Gal4* are stained with anti-Eve. (A) Wild-type embryo. (B) *pbl*^{3} mutant embryo. (C) Expression of *Pbl*$^{\Delta C\text{-}term}$ in a *pbl*^{3} mutant embryo results in the increase of the amount of Eve positive cell clusters (arrows). (D) Expression of *Pbl*$^{\Delta PH}$ does not change the number of Eve positive cell clusters in *pbl*^{3} mutants (arrow).
(E-G) Expression of *Pbl*$^{\Delta C\text{-}term}$ and *Pbl*$^{\Delta PH}$ with *twi::Gal4, twi::CD2* in *pbl*^{3} mutant embryos. Mesoderm cells are marked with anti-Twi (green) and anti-CD2 (red). (E) Mesoderm cells in an embryo expressing *twi::CD2* in wild-type background. (F) Expression of *Pbl*$^{\Delta C\text{-}term}$ in *pbl3* mutant embryos is not able to rescue cytokinesis defects. Binuclear cells are found in the mesoderm (arrow). (G) *pbl*^{3} mutant embryos expressing *Pbl*$^{\Delta PH}$ show defects in cytokinesis (arrow).

Since Pbl is also required for the formation of the contractile ring during cytokinesis, we examined the function of the C-terminal tail and the PH domain by testing the rescue ability of *Pbl*$^{\Delta C\text{-}term}$ and *Pbl*$^{\Delta PH}$ in cytokinesis. To investigate if cells

undergo cytokinesis a cell surface marker was used. The transgene *twi::CD2* encodes the CD2 transmembrane protein from rat in all mesoderm cells (Fig. 4.3 E, F, G). CD2 localizes to the plasma membrane and can be detected with a CD2 specific antibody (Fig. 4.3 E, F, G). In the wild type mitosis is followed by cytokinesis when the cytoplasm and nucleus of the daughter cells are separated by cell membrane (Fig. 4.3 E). In pbl^3 mutant embryos cytokinesis is defective and after mitosis the daughter-nuclei stay together in a common cytoplasm *(Prokopenko et al., 1999;* see chapter 1.6*)*. The two transgenes, $Pbl^{\Delta C\text{-}term}$ and $Pbl^{\Delta PH}$ failed to rescue cytokinesis defects. Binuclear cells are found in pbl^3 mutant embryos expressing $Pbl^{\Delta C\text{-}term}$ and $Pbl^{\Delta PH}$ (Fig. 4.3 F, G).

Theses experiments showed a potential role for the C-terminal tail and the PH domain for Pbl function in cytokinesis.

4.3 Localization of the C-terminal tail and the PH-domain in htl mutants

In summary, the previous results revealed that presumably without localization at the cell cortex Pbl is not able to function properly during cell migration. The mechanisms that are required for the localization of Pbl are not clear yet. However the results in this thesis so far (chapter 4.1 and 4.2) reveal roles for the C-terminal tail and the PH domain in cortical localization of Pbl.

The key question is how the localization of Pbl via the C-terminal tail and the PH domain is regulated. Our model implies a role of the FGF receptor Htl in regulation of Pbl, since Htl is required for the migration and differentiation of the mesoderm *(Frasch et al., 1987; Beiman et al., 1996; Michelson et al., 1998; Shishido et al., 1993; Shishido et al., 1997).* According to the function of Pbl in the reorganization of the actin cytoskeleton and the similar phenotype to *htl* mutants, Pbl could be directly regulated through the FGF signalling pathway. It was indeed shown, that the activity of Pbl is

required for the function of Htl in the regulation of cell shape changes. The expression of Htl and λHtl, a constitutive active form of the receptor, were unable to induce cell shape changes in early *pbl* mutant embryos. These results show that Pbl is required for the early cell shape changes triggered by Htl *(Schumacher et al., 2004).*

To investigate whether Pbl's subcellular localization is regulated through Htl signalling transgenes encoding for various Pbl constructs were expressed in *htl* mutant embryos. $Pbl^{full\text{-}length}$ is localized normally in *htl* mutant embryos and the expression of $Pbl^{full\text{-}length}$ is not able to rescue the *htl* mutant phenotype (*van Impel, unpublished*). To investigate if Htl signalling is required for the localization of Pbl at the cell cortex through the C-terminal tail or the PH domain, each of the domains was expressed in *htl* mutant embryos. As already mentioned the C-terminal tail is restricted to the cytoplasm and the cell cortex in mesoderm cells (Fig. 4.4 A, B). In *htl* mutant embryos the C-terminal tail is localized at the cell cortex and the cytoplasm, however there is more protein present in the cytoplasm compared to wild type. The localization to the cell cortex does not seem as prominent as in wild type (Fig. 4.4 C, D).

In initial experiments, the localization of the PH domain was analyzed by expression of PH-GFP. The GFP signal was too weak to consider the result. Therefore the localization of the $Pbl^{DH\text{-}PH\text{-}HA}$ transgene was analyzed. Since the DH domain alone does not localize to the cell cortex the PH domain is required for cortical localization, so the $Pbl^{DH\text{-}PH\text{-}HA}$ expression mimics the localization of the PH domain. The localization of $Pbl^{DH\text{-}PH\text{-}HA}$ in *htl* mutants (Fig. 4.4 G, H) is similar as in wild type (Fig. 4.4 E, F). In both genetic backgrounds the $Pbl^{DH\text{-}PH\text{-}HA}$ is localized to the cell cortex.

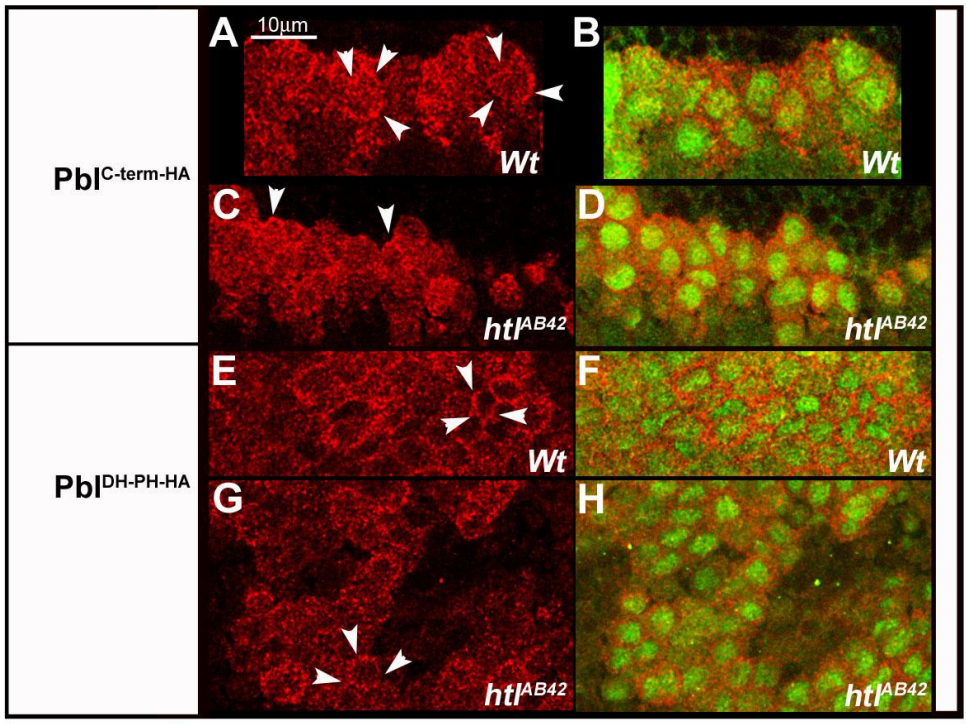


Fig. 4.4 Localization of the C-terminal tail and the PH domain at the cell cortex of mesoderm cells in *htl* mutant embryos.
Mesoderm cells are stained with anti-HA (red) and anti-Twi (green). (A, B) The C-terminal tail is restricted to the cell cortex in wild type background (arrows). (C, D) In htl^{AB42} mutant embryos the C-terminal tail is localized to the cell cortex (arrows), but not as focused as in wild type. More protein localizes to the cytoplasm. (E, F) $Pbl^{DH\text{-}PH\text{-}HA}$ is restricted to the cell cortex in wild type (G, H) and in htl^{AB42} mutant embryos (arrows).

These results reveal a possible role for Htl signalling in regulation of Pbl via the C-terminal tail, whereas the localization of Pbl through the PH domain does not seem to be regulated by Htl signalling.

4.4 Conclusions

The investigation of $Pbl^{\Delta C\text{-}term}$ and $Pbl^{\Delta PH}$ determined important roles for the C-terminal tail and the PH domain for the localization and function of the full length Pbl protein during mesoderm migration. Since the $Pbl^{\Delta C\text{-}term}$ protein was still able to localize to the cell cortex and could rescue migration to some extent, it might play a regulatory role in mesoderm migration through Htl signalling. The PH domain seems to be sufficient for cortical localization, but since the amount of protein at the cell cortex is very low, the C-terminal tail might be required for anchoring or stability of the protein at the cell cortex. Consistent with this interpretation the trangene $Pbl^{\Delta PH}$ was not

restricted cortically. Even though the C-terminal tail is present, it is not sufficient to place the protein to the cell cortex, giving the PH domain an essential role in cortical localization. This cortical localization might be independent from Htl signalling, because the PH domain was restricted normally in the absence of Htl receptor. The C-terminal tail is required for cortical localization as well. It is possible, that the C-terminal tail is the domain of Pbl, which interacts with Htl signalling, since it seemed to be not localized properly in *htl* mutants.

4.5 Analysis of Serine^{825} in the C-terminal tail

The data so far suggest that C-terminal tail of Pbl is required for its localization and function during mesoderm migration and likely to be regulated through Htl signalling. Furthermore the C-terminal tail is essential for the function, but not the localization of Pbl during cytokinesis *(van Impel et al., 2009).*

In addition to its function in localization, the C-terminal tail is important for the regulation of substrate specificity of Pbl for the GTPases Rac and Rho. The transgene encoding $Pbl^{\Delta N\text{-}term}$, which differs from $Pbl^{DH\text{-}PH}$ in the presence of the C-terminal tail cannot rescue mesoderm migration defects, furthermore it causes defects during cytokinesis and invagination in the embryo (*van Impel et al., 2009)*. Usually *pbl* mutants do not exhibit defects in internalization of the mesoderm; therefore these dominant defects suggest an abnormal activity of $Pbl^{\Delta N\text{-}term}$. Over-expression of $Pbl^{\Delta N\text{-}term}$ seems to activate Rho1 during invagination of the mesoderm ectopically; usually Rho1 is activated by RhoGEF2 in this process *(Barrett et al., 1997).*

$Pbl^{DH\text{-}PH}$ lacking the C-terminal tail was able to rescue the mesoderm migration defects in pbl^{3} mutant embryos to some extent. Expression of $Pbl^{DH\text{-}PH}$ in wild type embryos causes defects in mesoderm migration but it does not affect cytokinesis. The different dominant misexpression phenotypes of both gain of function forms of Pbl

suggest an important role for the C-terminal tail for the regulation of the specificity tandem DH-PH domain of Pbl for Rac and Rho *(van Impel et al., 2009).*

How is Pbl localization and exchange activity regulated through the C-terminal tail?

The C-terminal tail contains evolutionary conserved phosphorylation sites (Fig. 4.6). An attractive model is that Htl signalling is required for phosphorylation of the C-terminal tail of Pbl and thereby triggering the change of the guanine nucleotide exchange activity of Pbl. Serine^{825} in the C-terminal tail is highly conserved and was identified by phospho-mass-spectrometry analysis to be phosphorylated (Fig. 4.5; Phosphopep; *ISB Home, http://www.phosphopep.org/index.php).* To investigate the consequences when Ser^{825} in the C-terminal tail of Pbl cannot be phosphorylated, a transgene was created where Ser^{825} is mutated into Ala. The localization and function of this protein was investigated.

Identifed Sequence	PeptideProphet	Tryptic Ends	Peptide Mass	DeltaCN	# Obs	# Mappings	Links
R.RDS*LPISFNKR.K	1.00	2	1412.71	0.42	2	1/4	
R.RDS*LPISFNK.R	0.99	2	1256.60	0.29	9	1/4	
R.AFS*FNKT*PNK.L	0.98	2	1313.54	0.23	4	1/4	
R.AFS*FNKTPNK.L	0.84	2	1233.58	0.16	1	1/4	
K.GFNTAKS*PSTAK.T	0.96	2	1288.58	0.14	2	1/4	
R.RSS*VSDAGLLSVSNSLFDCTTSPDKLESDK.L	0.96	2	3238.42	0.07	1	1/4	
R.AVS*TMMTSPFGSTNSLTPASQLAQMR.L	0.92	2	2794.24	0.06	2	1/4	
R.SSS*PSTQSEMLVVPPLSVQPTRK.N	0.84	2	2535.22	0.04	1	1/4	
R.AVSTMMTSPFGS*TNSLTPASQLAQMR.L	0.89	2	2794.24	0.01	1	1/4	
R.SSSPS*TQSEMLVVPPLSVQPTR.K	0.99	2	2407.12	0.01	12	1/4	
V.PPLSVQPTR.K	0.97	1	994.58	0.39	2	1/4	
K.LHANWR.E	0.87	2	796.44	0.13	1	1/4	

Fig. 4.5 Phosphorylation of Ser825 was predicted by phospho-mass-spectrometry.
Phosphopep; *ISB Home, http://www.phosphopep.org/index.php)*

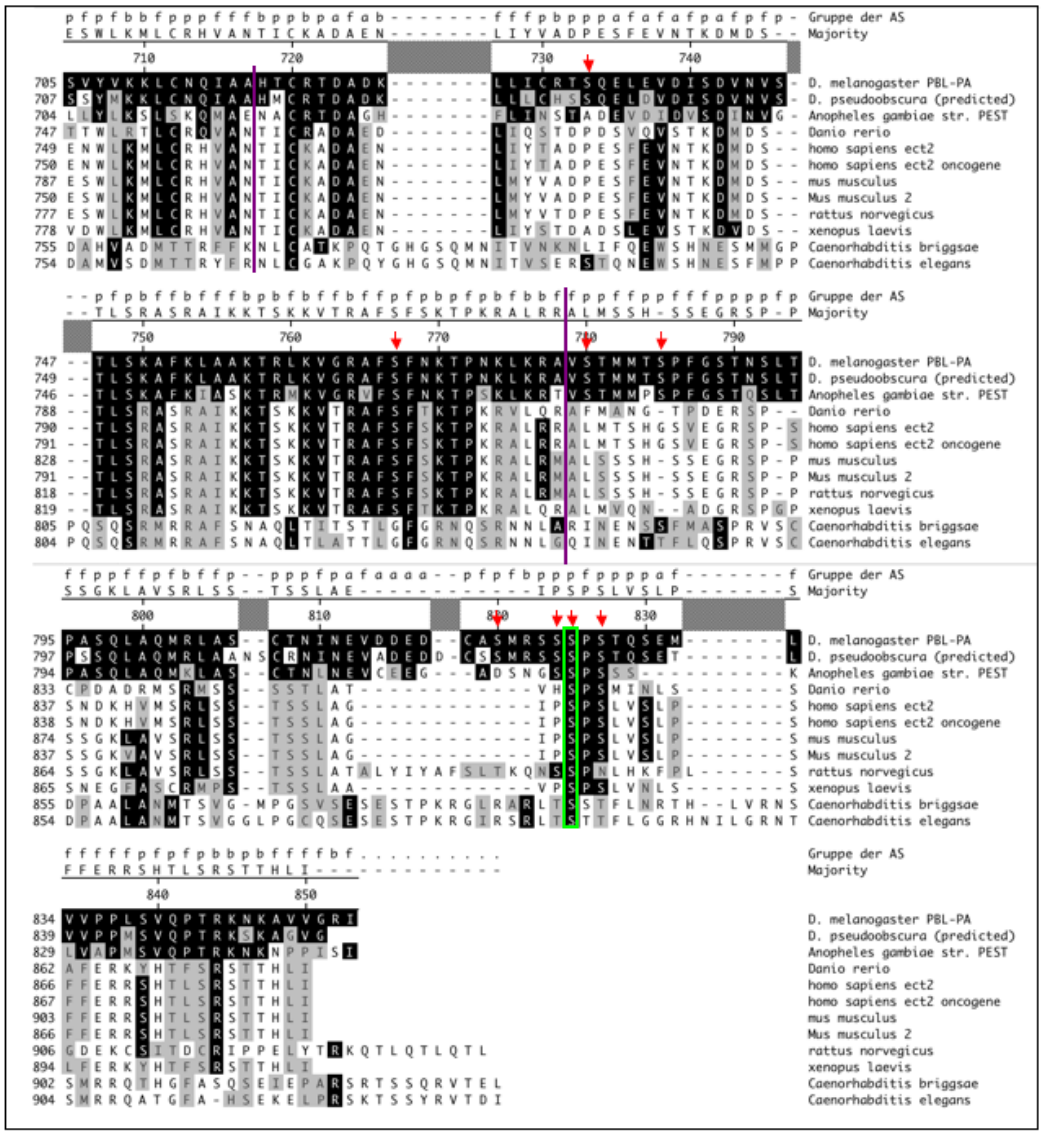


Fig. 4.6 Ser^{825} is conserved among different species.
The sequence of the C-terminal tail of Pbl contains many conserved Serines (arrows), which might be potential phosphorylation sites. Ser^{825} (green box) is highly conserved and was preidcted to be phosphorylated. The sequences between the purple lines was shown not to be important for the function of the C-terminal tail (*van Impel et al., 2009*).

4.5.1 Phosphorylation of Ser^{825} is not required for normal localization of Pbl

Pbl$^{S825A\text{-}HA}$ was expressed with *twi::Gal4* in the mesoderm of wild type embryos and the localization was analyzed by antibody staining for the tagged protein. The localization of *Pbl$^{S825A\text{-}HA}$* was similar to *Pbl$^{full\text{-}length\text{-}HA}$* (Fig. 4.7 A, B, E, F), but the amount of protein at the cell cortex seems lower compared to *Pbl$^{full\text{-}length\text{-}HA}$* (Fig. 4.7 E,

F). The mutation of the Serine site did not affect the localization of the protein as strong as the absence of the C-terminal tail.

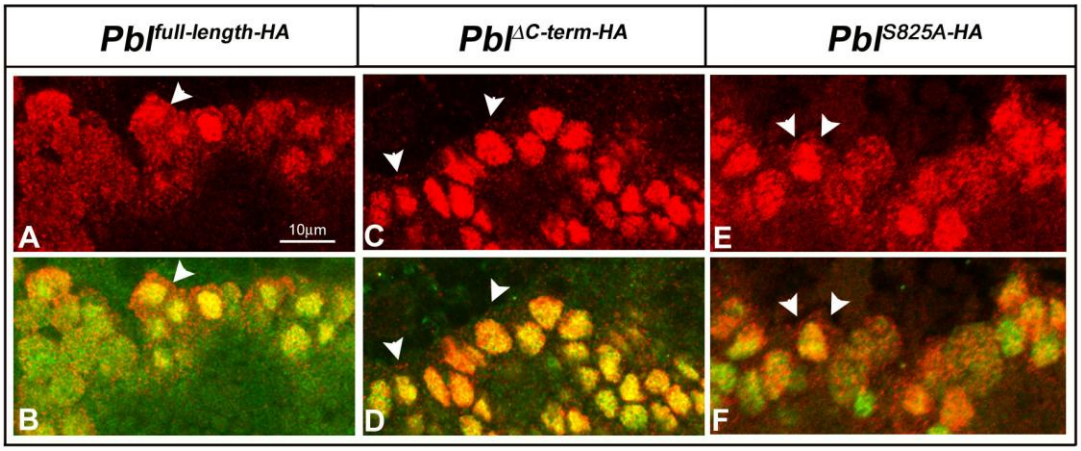


Fig. 4.7 Localization of *Pbl$^{\Delta C\text{-}term\text{-}HA}$* and *Pbl$^{S825A\text{-}HA}$* in mesoderm cells.
Embryos expressing *Pbl$^{full\text{-}length\text{-}HA}$*, *Pbl$^{\Delta C\text{-}term\text{-}HA}$* and *Pbl$^{S825A\text{-}HA}$* using *twi::Gal4* in mesoderm cells are stained with anti-Twi (green) and anti-HA (red). (A, B) Expression of *Pbl$^{full\text{-}length\text{-}HA}$*. (C, D) *Pbl$^{\Delta C\text{-}term\text{-}HA}$* localization in migrating mesoderm cells. (E, F) *Pbl$^{S825A\text{-}HA}$* is expressed in the nucleus and localized in the cytoplasm and the cell-cortex. The amount of *Pbl$^{S825A\text{-}HA}$* protein in the cytoplasm and the cortex is higher than of *Pbl$^{\Delta C\text{-}term\text{-}HA}$*, it is similar to *Pbl$^{full\text{-}length\text{-}HA}$*.

The phosphoryaltion of Ser^{825} is probably not required for localization of Pbl during mesoderm migration. Other sequences in the C-terminal tail must play a role for localization and stability of the protein which are functional in the *Pbl$^{S825A\text{-}HA}$* protein.

4.5.2 Phosphorylation of Serine^{825} in the C-terminal tail is important for the function of Pbl during mesoderm migration

To investigate whether mutation of the phosphorylation site Ser^{825} affects the function of Pbl in mesoderm migration the transgene *Pbl^{S825A}* was expressed in *pbl^{3}* mutant embryos. The rescue ability *of Pbl^{S825A}* was investigated by quantifying the number of Eve positive cell clusters. The number of Eve clusters after expression of *Pbl^{S825A}* in *pbl^{3}* mutant embryos with a mean value of 10,9 was significantly increased compared to *pbl^{3}* mutant embryos. Interestingly, these results demonstrate that the

Pbl^{S825A} protein cannot fully rescue migration defects (Table 4.2; Fig. 4.8 C, D), indicating a role for this phosphorylation site for Pbl function.

genotype	Eve positive hemisegments	S.D.	n
***pbl*^{3}/*pbl*^{3}**	**1,7**	**1,7**	**128**
Pbl$^{full\text{-}length\text{-}HA}$; *pbl*^{3}/*pbl*^{3}	**18,6**	**1,7**	**98**
Pbl$^{\Delta C\text{-}term\text{-}HA}$; *pbl*^{3}/*pbl*^{3}	**5,3**	**1,0**	**196**
Pbl$^{S825A\text{-}HA}$; *pbl*^{3}/*pbl*^{3}	**10,9**	**1,6**	**126**

Table 4.2 Average numbers of Eve positive cell clusters in *pbl*^{3} mutant background after expression of *Pbl*$^{\Delta C\text{-}term}$ and *Pbl*^{S825A}.
The table shows mean values of Eve positive hemisegments, the standard deviation (S.D.) and the number of embryos (n) analyzed.

To analyze the cytokinesis defects the embryos were stained with DAPI to mark the nuclei. The nuclei in *pbl*^{3} mutant embryos are bigger compared to wild type embryos (Fig. 4.8 E, F), because of the failure in cytokinesis. After expression of *Pbl*^{S825A} with *twi::Gal4* in *pbl*^{3} mutant embryos the cytokinesis defects are suppressed in the mesoderm cells (Fig. 4.8 C, D), but not in the rest of the embryo (Fig. 4.8 G).

These data indicate a role for phosphorylation of Ser^{825} of Pbl during mesoderm migration. However Ser^{825} is not required for Pbl localization and function in cytokinesis.

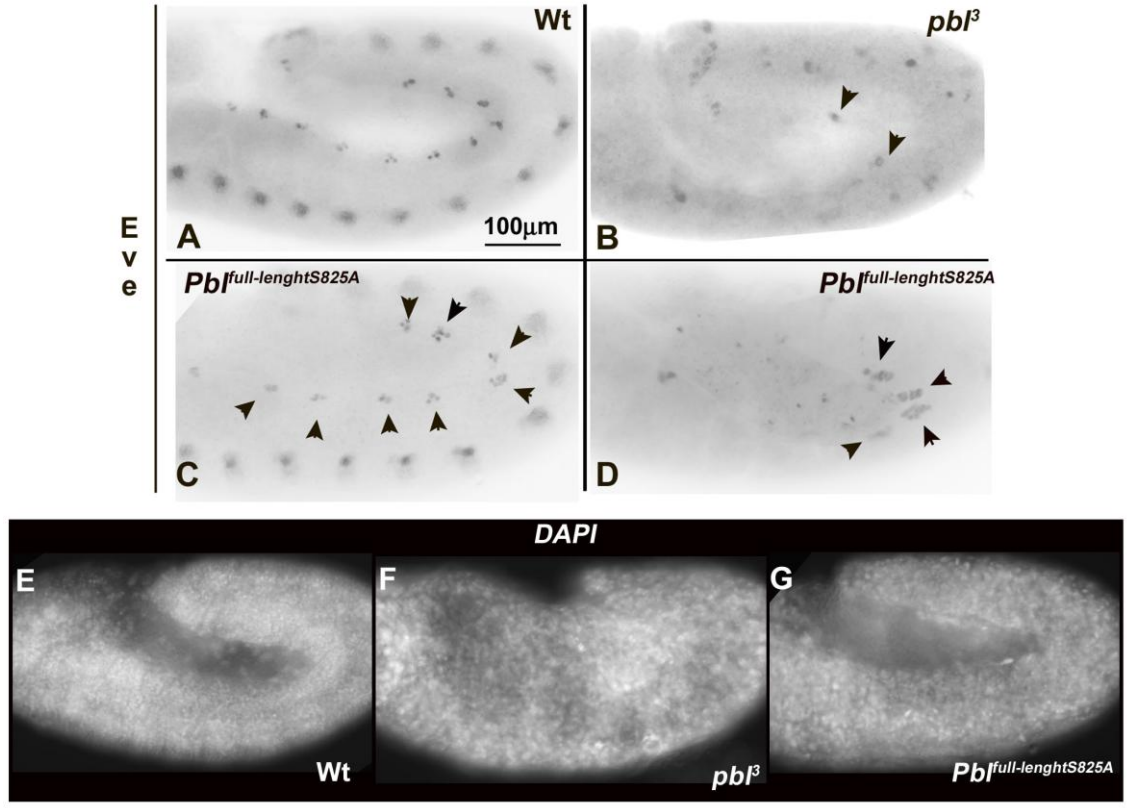


Fig. 4.8 Rescue ability of *Pbl*^{S825A} in *pbl*^{3} mutant embryos.
(A-D) Embryos expressing *Pbl*^{S825A} with *twi::Gal4* are stained with anti-Eve (A-D) and DAPI (E-G). (A) Wild type embryo. (B) *pbl*^{3} mutant embryo. (C, D) Expression of *Pbl*^{S825A} in *pbl*^{3} mutant embryos results in the increase of the amount of Eve positive cell clusters (arrows). Furthermore the size of the nuclei is comparable to wild type, but there are more cells in some clusters. (E) Wild type embryo, the nuclei are stained with DAPI. (F) The nuclei in *pbl*^{3} mutant embryos are larger, due to defects in cytokinesis. (G) Multinucleated cells are observed in embryos expressing *Pbl*^{S825A}.

4.5.3 Conclusions of 4.5

The C-terminal tail is an important domain for the regulation of Pbl function during mesoderm development and cytokinesis (this work chapter 4*; van Impel et al., 2009).* The analysis of the Ser^{825} revealed a possible role for this site in the regulation of Pbl. Mutation of Ser^{825} into Ala, which cannot be phosphorylated interferes with the function of this protein during mesoderm cell migration. Although the protein carrying this mutation is able to rescue mesoderm migration defects in *pbl*^{3} mutant embryos to some extent, the suppression is lower compared to the rescue with *Pbl*$^{full\text{-}length}$. However the phosphorylation of this site is not important for proper localization of the protein, although the amount of protein outside the nucleus seems to be lower compared to

Pbl$^{full\text{-}length}$. Therefore other sequence motifs will have to be considered to explain the localization to the cell cortex. Ser^{825} is probably not required for cytokinesis, because the nuclei in Eve positive cells did not seem to be multinuclear.

5 Discussion

The RhoGEF Pbl is required for cell shape changes and the formation of cellular protrusions (*Schumacher et la., 2004; Smallhorn et al., 2004*) during FGF signalling regulated mesoderm cell migration (*Frasch et al., 1987; Beiman et al., 1996; Michelson et al., 1998; Shishido et al., 1993; Shishido et al., 1997*). However the molecular mechanisms underlying mesoderm cell migration via Htl and Pbl are unknown.

To identify members of the FGF-Pbl signalling pathway an eye modifier screen was performed using a gain of function form of Pbl. Several candidates where found that interact with Pbl including seven mutants, which reveal defects in mesoderm migration. Furthermore experiments in this work revealed essential function for the PH domain in localization at the cell cortex and exchange activity of Pbl independent of Htl signalling. Additionally the C-terminal tail is required for localization at the cell cortex and function of Pbl during mesoderm migration and cytokinesis. Furthermore the localization of the C-terminal tail seems to be regulated through Htl signalling and its function might be regulated by phosphorylation of Ser^{825}.

The results found in this work are going to be discussed here and furthermore a model for mesoderm cell migration regulated by Pbl and Htl will be presented.

5.1 Genetic interactors of Pbl were found in eye modifier screens

To find genes that interact with Pbl in FGF regulated mesoderm migration a genetic screen was performed. In *Drosophila,* genetic screens are widely used to find new genes acting in cell signalling pathways. Usually in a genetic screen the gene function is reduced by mutation, deletion or RNAi and a particular phenotype is screened. The mutated genes causing the phenotype are mapped to a cytological region and the function of the gene is then characterized on many levels. A very important screen was carried out by Christiane Nüsslein-Vollhard and Eric Wieschhaus in the

1970s. They found many important genes, which are required for *Drosophila* embryogenesis. These findings were honored with the Nobel price for physiology or medicine in 1995.

In the genetic screen carried out in this work a gain of function form of Pbl was used. A constitutive activation of Rho GEF's can be achieved by truncation of the amino-terminal part of the protein (*Whitehead et al. 1997*). In Pbl and its ortholog Ect2, the lack of the N-terminal regulatory domains, BRCT1 and 2, PEST and NLS domain, results in a constitutive activation of the proteins (*Miki et al., 1993; Prokopenko et al., 1999*).

The constitutively active form $Pbl^{DH\text{-}PH}$ was expressed in the compound eyes of flies, carrying chromosomal deletions and EMS induced point mutations. The compound eye is a commonly used model to test genetic interaction in vivo. The eye is very sensitive to changes of protein expression and shows morphological defects. The same gain of function form of Pbl was used in a similar eye modifier screen already, where Rho1 was found being a substrate of Pbl (*Prokopenko et al., 1999*). The screens described above are so called forward genetics screens, meaning a particular phenotype is searched and afterwards the gene is going to be mapped. Compared to the forward genetics there is reverse genetics, where a known gene is mutated and the resulting phenotype is going to be investigated. An example for a reverse genetics screen was done with Pbl by the Saint lab in 2007. $Pbl^{\Delta DH}$, a Pbl transgene without exchange activity, was used to find interactors of Pbl during cytokinesis. $Pbl^{\Delta DH}$ expression with *GMR::Gal4* in the eye results in a reduced activation of Rho1 during cytokinesis causing the remarkable rough eye phenotype. Different genes were overexpressed with *GMR::Gal4* and in case of acting in this pathway modified the rough eye phenotype. 112 genes were found interacting with Pbl during cytokinesis (*Gregory et al., 2007*). The genes found during that screen were divided into three classes: cell cycle genes,

signalling effectors and metabolic enzymes. A set of genes were found, that are involved in cytoskeletal signalling, including microtuble binding proteins. Some of the genes are required for cell migration, including Rac2, Rap1 and members of the Wg/Wnt signalling pathway. This type of screening is quite useful because it avoids the drawn-out processes of mapping. On the other hand the amount of genes with inserts required for over-expression of the genes is limited.

A number of screens to find genes that are involved in mesoderm migration by looking at the zygotic and maternal phenotype were carried out in our lab before (*Gryzik and Müller, 2004*). The modifier screen performed in this thesis is a more sophisticated way to directly look for genes that specifically interact with Pbl.

5.1.1 The modifier screen with chromosomal deletions defined regions containing genetic interactors of Pbl

The modifier screen performed in this work revealed regions on the second and third chromosomes containing genes, which genetically interact with Pbl. However the mapping for single genes based on the breakpoints of chromosomal deletions turned out to be difficult. Loss of function mutations in the genes localized to the modifier regions were required to test interaction of these genes with Pbl and to investigate these genes for defects in cell migration. Mutations and P-element insertions in various genes localized to the modifier regions were tested. Only a few candidates showed genetic interaction with Pbl in the eye. Some of the potential interactors function in actin-cytoskeleton processes or in mesoderm development. Though most genes found in the screen are required for transcriptional processes. We expected to find interactors of Pbl during mesoderm migration that regulate Pbl activity and localization post-translational,

for the reason that mesoderm migration is a rapid process without transcription and Pbl is expressed in the nucleus in high amounts a regulation of Pbl activity by transcription is unlikely. Nevertheless these genes could give insight into other processes Pbl might be involved in, for example the role of Pbl in the nucleus is still unknown. Pbl contains BRCT domains, which are commonly found in proteins involved in cell cycle control and DNA damage, Pbl might have a function in these processes. More regions were found, which show genetic interaction with Pbl, but could not be mapped more accurately. These regions might contain more interesting candidates.

5.1.2 Genetic modifiers found using chemical mutagenesis

The deletion modifier screen revealed candidates for an interaction with Pbl. The limited amount of small deletions and loss of function mutations made it difficult to investigate all modifier regions. Therefore a second screen was designed using EMS induced mutations. 23 mutations on the second and third chromosomes, which interact with Pbl genetically, were found; seven of them are required for mesoderm cell migration.

EMS is a chemical mutagen, which can change the characteristics of DNA and cause mutations that influence gene activity. The mutations caused by EMS are loss of function mutations with a high frequency, yet there is a disadvantage of EMS inducing point-mutations randomly and in more than one gene on the same chromosome.

The modification of the rough eye phenotype was investigated in the F1 progeny of the mutated flies already. This means after only one generation (development of the flies at 22°C takes ~14-17 days) eye modifiers can be selected. Therefore the mutagenesis and screening was completed very quickly. A large number of suppressors was selected, but after retesting only a small number of suppressors remained. The reason for this is in screening and selecting for single males that suppress the rough eye phenotype. After crossing these males to females with chromosomal markers

homozygous lethal mutations were selected and stocks were established. The number of candidates was reduced after the selection of homozygous lethal stocks, therefore it is concluded that most of the modifiers are homozygous viable mutations. Finally in the retest only stocks were selected, where the suppression is highly penetrant, meaning every fly with the same genotype shows suppression. The number of candidates that remained in the end is similar to the number of modifier regions found in the initial screen, so hopefully after complementation studies with the deletions the mutations can be assigned to a defined region. Afterwards the mutations are going to be mapped by sequencing. Some of the mutations could be defined to one chromosome arm already after the generation of germ line clones since every chromosome arm contains FRT sites. The induction of germ line clones showed that five mutations, which did not display mesoderm migration defects in zygotic mutants have maternal contribution. They exhibited defects in mesoderm migration, when the maternal gene product was removed. In 13 mutations the recombinant females did not lay eggs, indicating an essential function of the mutated gene in oogenesis. Since Rac and Rho1 are required for border cell migration during oogenesis (*Duchek et al., 2001*) the genes might interfere with Rac or Rho processes. The migrating border cells express PVR and expression of PDGF/VEGF by the oocyte guides the migrating border cells. PVR activates Rac which induces actin polymerization and the formation of cellular protrusions (*Duchek et al., 2001*). Pbl germline clones do not show any defects and therefore a function of Pbl during oogenesis can be excluded. On the other hand the number of mutated recombination lines that did not lay eggs was quite large, therefore it is possible that the recombination did not work properly in some flies and should be repeated for some mutants.

Two zygotic mutants showed defects in mesoderm migration. However the defects were variable in embryos of both mutants. It might be that the EMS mutation

does not result in loss of function of the protein. Besides it is possible that the mutated genes have maternal contribution and there is still functional gene product in the embryo consequently the phenotype is variable. For that reason germline clones for the two mutants Su(3)29-10-3 and Su(3)31-10-1 were generated.

Female mosaics with the mutation Su(3)29-10-3 did not lay any eggs. By recombination the sequences of both chromosome arms were exchanged and mutations on both chromosome arms were revealed. These mutations could have influenced each other and the mesoderm phenotype. Only one mutation showed suppression of the rough eye phenotype, it is on the left arm of the third chromosome. The female mosaics with the mutation Su(3)31-10-1 on the left arm did not show any defects. Females with the recombination on the right arm laid a small number of eggs, some embryos displayed defects, but it is difficult to make a statement whether the defects are prominent or not and therefore more germline clones have to generated and embryos analyzed.

The EMS modifier screen revealed seven mutations that interact genetically with Pbl and show defects in mesoderm development. The mapping and characterization of the affected genes has the potential to give more insight into Pbl function. Surprisingly most mutations are located on the left arm of the third chromosome. This region was not mapped very accurately in the initial screen due to unavailability of small deletions. Pbl is mapped to the left arm of the third chromosome (66A18-19) but Pbl complements the two zygotic mutations, meaning that *pbl* is unaffected in Su(3)31-10-1 and Su(3)29-10-3. Additionally complementation studies were performed with *htl* and *dof*, which are on the third chromosome as well and revealed that these genes are not affected. Rac2 and Nesthocker, both required for mesoderm migration, are located on the third chromosome as well and complementation needs to be tested. It seems that the third

chromosome contains a high number of genes involved in mesoderm cell migration and possibly we found some more genes in the screen interacting in this process.

5.1.3 Strengths and limitations of the modifier screen

The use of Pbl gain of function in the eye was an advantage in this screen, because we could find genes that genetically interact with Pbl. We know that the genetic interaction with Pbl and the genes found in the screen is specific, because previous work showed that modification of the rough eye phenotype is not observed in mutants of other GTPases than Rho and Rac. The structure similarities of DH domains in GEFs are very low, so the interaction of DH domains with the switch regions of GTPases is very specific (*Rossman et al., 2005*). Furthermore to test interaction in the eye is much simpler and quicker, than to test in the embryo. Modifier analysis in embryos could be carried out as well, since the $Pbl^{DH\text{-}PH}$ over-expression causes defects in mesoderm migration. However the collection, fixation and stainings of embryos would take much longer, than to screen the eyes of flies.

One disadvantage of the modifier screen was that the modifiers found are not involved in mesoderm development for sure, but might interact with Pbl during other processes for example cytokinesis. To separate Pbl interactors in cytokinesis we could look for modification of the rough eye phenotype after expression of $Pbl^{\Delta N\text{-}term}$ in the eye. $Pbl^{\Delta N\text{-}term}$ causes morphological defects in the eye as well and in the embryo $Pbl^{\Delta N\text{-}term}$ interferes with Rho1 dependent processes, cytokinesis and invagination only (*van Impel et al., 2009*). For that reason modification of the found $Pbl^{DH\text{-}PH}$ interactors, which also show genetic interaction with $Pbl^{\Delta N\text{-}term}$ would indicate an interaction with Pbl in cytokinesis. Another disadvantage is the temperature sensitivity of Gal4 expression. At lower temperatures only small amount of Gal4 is expressed, therefore the amount of transgene expression is low as well and the defects in the eye are weak. At higher temperatures more Gal4 is expressed, more transgene is expressed and the

defects are stronger. Therefore the flies are kept in an incubator with a constant temperature of 22°C, but still not all flies showed the same degree of defects. An unstable temperature in the incubator could lead to this differences. Another reason might be that the individual flies react differently to the over-expression of $Pbl^{DH\text{-}PH}$. For that reason the identification of modifiers was difficult sometimes. From a group of flies with the same genotype, some flies showed modification and others did not. To solve this problem the crosses were repeated two-three times until the modification was obvious in all flies of the same genotype. The flies were always compared to control flies, which express $Pbl^{DH\text{-}PH}$ in the original line without mutation.

The modifier screens turned out to be an excellent way to find new genes that genetically interact with Pbl. In both screens interactors of Pbl were found, which are involved in mesoderm migration. Furthermore genes were identified that could give insight into other functions of Pbl as well. In the EMS screen two candidates were found, that genetically interact with Pbl and are involved in mesoderm migration. These genes are going to be mapped and characterized. Additionally genes were found, which have maternal function and show defects in oogenesis and mesoderm migration.

5.2 The C-terminal tail and the PH domain are required for localization and function of Pbl

Investigation of the PH domain and the C-terminal tail of Pbl revealed important roles for both domains in cortical localization and function of Pbl in mesoderm migration and cytokinesis. Without these domains Pbl is not localized properly and cannot induce cell migration. In the next paragraphs the possible functions of the PH domain and the C-terminal tail will be discussed.

5.2.1 The localization and function of Pbl during mesoderm cell migration depends on the PH domain

In this work it was shown for the first time that the PH domain is required for cortical localization of Pbl. A Pbl protein lacking the PH domain is absent from the cell cortex. Whereas a protein missing the C-terminal tail results in a decrease of protein outside the nucleus, but the protein is still localized to the cell cortex, which presumably is mediated via the PH domain. The PH domain and the C-terminal tail are the only domains that localize to the cell cortex. It was shown that the DH domain and the BRCT domains did not localize cortically in interphase cells (*van Impel et al., 2009)*. For Ect2, the human orthologue of Pbl, it was revealed before that the PH domain is required for cortical localization of Ect2 in interphase cells *(Chalamalasetty et al., 2006)*.

PH domains of DH domain family GEFs are known for binding to phospholipids in cell membranes *(Rossman et al., 2005)*. Phosphoinositides, especially PI(3,5)P2 and PI(3,4,5)P3 play essential roles in the regulation of actin cytoskeleton organization and dynamics and are therefore important for many processes including cytokinesis and cell migration *(Saarikangas, et al., 2010)*. Many GEFs are regulated by PI(3,4,5)P3 through binding of their PH domain *(Das et al., 2000; Sasaki et al., 2006; Welch et al., 2002)*. The broadly expressed GEF Vav2 for example is activated through direct binding of PIP2 and PIP3 to its PH domain *(Tamás et al., 2003)*.

Consequently a potential function of the PH domain of Pbl could be a specific binding to phospholipids, like for example phosphoinositides. A role for phosphoinositides in regulation of cell migration via Rac GTPase was shown before. Rac is regulated by PI3K signalling in many organisms. PI3K and Rac interact during the formation of membrane ruffles induced by IGF signalling and PI3K is acting upstream of Rac during PDGF induced cytoskeleton changes and lamellipodia formation *(Kotany et al., 1994; Hawkins et al., 1995; Wennström, 1994)*. Moreover Rac

is required for the synthesis of PIP2 at the plasma membrane *(Chatah and Abrams, 2001; Saarikangas et al., 2010)* and Rac can bind to and activate PI3K, so it is possible that a feedback loop exists *(Hawkins et al., 1995, Bokoch et al., 1996)*.

PH domains can also bind to other proteins rather than phospholipids. For example, the PH domain of the GEF Dbl binds to Ezrin, a protein, which is involved in linking the plasma membrane and the cytoskeleton *(Vanni, et al., 2004)*. Another example is the PH domain of the GEF Trio1 as it directly binds to Filamin, a protein for cross-linking filamentous actin *(Bellanger et al., 2000)*.

It will be important to investigate the binding specifity of the PH domain either to phosphoinositides or other proteins. One possibility would be to generate a PH-GST fusion protein and to identify proteins that bind to the PH domain by affinity chromatography. This can lead us to a better understanding about how Pbl is regulated through FGF signalling or maybe other, parallel pathways like for example PI3K/Akt. It is unknown if Htl signalling activates PI3K pathway, which is commonly done by FGF signalling. However in *Drosophila* PI3K /Akt pathway is required for follicle cell growth in oogensis and in PI3K mutants ooegensis is blocked; therefore a function in mesoderm migration cannot be investigated in PI3K mutants. One way to examine a possible function for PI3K in mesoderm migration would be by analysis of hypomorphic alleles, mutations of the gene that alter the gene expression and do not fully reduce it. Aditionally overexpression of PI3K in the mesoderm could be analyzed.

Furthermore it is unclear if the localization of the PH domain is required for the function of Pbl or if the PH domain also supports the exchange activity of the DH domain. PH domains of GEFs are known to enhance the catalytic function of the DH domains by allosteric mechanisms *(Rossman et al., 2005)*. Either of the two roles of the PH domain of Pbl is possible. In some RhoGEFs swapping the PH domain for a membrane anchor restores their function; for example in Trio1 the PH domain is just a

protein interaction domain and does not regulate the exchange activity of the DH domain *(Seipel et al., 2001)*. On the other hand there are GEFs like Sos1, Dbs, Tiam1 and Vav1, where the binding of the PH domain to phospholipids is not specific and with a low affinity and thus other domains are required to regulate the cortical localization *(Rossman et al., 2005)*. However we do not know if the PH domain of Pbl acts as membrane anchor or supports the exchange activity of the DH domain or both.

To address this question an experiment where the PH domain of Pbl is exchanged for an unrelated, generic membrane anchor (ma) should be done. $Pbl^{\Delta PH\text{-}ma}$ should localize at the cell cortex via the membrane anchor, if $Pbl^{\Delta PH\text{-}ma}$ is able to rescue mesoderm migration defects in *pbl* mutant embryos, a function for the PH domain in supporting the DH domain can be excluded. On the other hand if the localization of $Pbl^{\Delta PH\text{-}ma}$ via the membrane anchor at the cell cortex would not rescue mesoderm migration, it would mean that the PH domain is not only essential for localization of Pbl but also for support of the catalytic function of the DH domain.

The investigation of the $Pbl^{\Delta PH}$ construct revealed that the PH domain is important for cortical localization and for the function of Pbl and it seems to be independent of Htl signalling.

5.2.2 The C-terminal tail of Pbl regulates its function and localization

The C-terminal tail of Pbl plays an important role for the function and the stability of Pbl. Although $Pbl^{\Delta C\text{-}term}$ is less abundant outside the nucleus compared to the amount of full-length protein, a protein without the C-terminal tail is still able to bind to the cell cortex and can rescue mesoderm migration to some extent. Therefore the C-terminal tail might have a role in stabilizing the protein outside the nucleus and/or to sustain the binding to proteins at the cell cortex. Moreover the C-terminal tail might

have an impact on the substrate specificity of Pbl for Rac and RhoA. The fact that the protein without the C-terminal tail can rescue migration to some degree leads to suggestions that this protein can activate Rac, but not strongly enough to fully rescue the migration defects. Whereas it cannot rescue cytokinesis although it is localized normally at the cleavage furrow (*van Impel et al., 2009*). Furthermore the gain of function $Pbl^{DH\text{-}PH}$ protein lacking the C-terminal tail interacts with both, RhoA and Rac in the eye; while the gain of function $Pbl^{\Delta N\text{-}term}$ protein, containing the C-terminal tail seems to interact with RhoA dependent processes only *(van Impel et al., 2009)*. These data strongly suggest a role for the C-terminal tail in substrate specificity. The exchange activity of $Pbl^{\Delta C\text{-}term}$ for Rac and RhoA needs to be tested, to see if there is a difference in activation of both.

In fact it was shown for the human orthologue Ect2 that the C-terminal tail regulates the GTPase specificity of the DH domain. However in mammalian cells the specificity switch appears to be the other way round. In Ect2 the DH-PH oncogenic form lacking the C-terminal tail interacts *in vivo* and *in vitro* with RhoA only. Whereas the oncogenic form DH-PH-C-term shows exchange activity for RhoA, Rac and Cdc42 *(Solski et al., 2004)*. Although the specificites are different, in both systems the C-terminal tail seems to regulate the switch between RhoA and Rac activation. In *Drosophila* mesoderm cells the switch might be regulated via Htl signalling, given that the C-terminal tail is mis-localized in the receptor mutant.

The work presented here and in van Impel et al (2009) provide the first hint for Pbl interaction with Htl signalling. In the future, protein interactions with the C-terminal tail should be defined to confirm the model that Pbl is regulated through its C-terminal tail by FGF signalling. In addition, the investigation of possible regulatory sequences in the C-terminal tail could give more insight into this.

5.2.3 Phosphorylation of Ser^{825} in the C-terminal tail is required for Pbl function

As already described the C-terminal tail of Pbl is important for localization and function of Pbl during mesoderm migration and cytokinesis. The function of Pbl might be regulated by Htl signalling via the C-terminal tail, which contains several phosphorylation sites.

Ser^{825} in the C-terminal tail is highly conserved and was predicted by the database ISB Phosphopep *(http://www.phosphopep.org/index.php)* to be an ambiguous phosphorylation site. Investigation of a protein where the Ser^{825} is mutated to an Alanine, which cannot be phosphorylated revealed a possible regulation of Pbl through phosphorylation. *Pbl*^{S825A} is localized similar to *Pbl*$^{full\text{-}length}$ in wild type embryos. The phosphorylation of Ser^{825} is not required for the localization of the protein at the cell cortex. The C-terminal tail probably contains other sequences that bind to the cell cortex either specifically or unspecifically. On the other hand the protein amount at the cell cortex is lower, which indicates a function for Ser^{825} in strengthening of the localization of the C-terminal tail at the cell cortex.

Furthermore the phospho-mutant *Pbl*^{S825A} protein cannot fully rescue the migration defects in Pbl mutant embryos. The amount of Eve cell clusters is higher than the amount after rescue with *Pbl*$^{\Delta C\text{-}term}$, but not as good as with *Pbl*$^{full\text{-}length}$ meaning that Ser^{825} is important for the function of Pbl in mesoderm migration. It is possible that phosphorylation of Ser^{825} in the C-terminal tail changes the exchange activity of the DH domain for Rac during mesoderm migration.

Phosphorylation of the Pbl ortholog Ect2 is required for its exchange activity during cytokinesis *(Tatsumoto et al., 1999)*. Indeed a phospho-mutation of T814A in the C-terminal tail of Ect2 slightly reduces the exchange activity of Ect2 for Rac *in vitro*.

The phosphorylation of another site T412 is required for the exchange activity for RhoA *(Niiya et al., 2006).*

Similar can be truth for Pbl. The phosphorylation might lead to a structure change of the C-terminal part of the protein, resulting in binding and activation of Rac and enhancement of the exchange activity of the DH domain. Without the phosphorylation, the DH domain can still bind to Rac and activate it, but possibly the binding or the exchange activity is not very strong and not sufficient to induce cell migration. Additionally the phosphorylation of Ser^{825} does not seem to be required for cytokinesis. Expression of Pbl^{S825A} is able to rescue cytokinesis in the mesoderm. Though these results have to be confirmed with stainings of rescued embryos with membrane markers.

Another, more simplier possibility for the lower rescue ability of Pbl^{S825A} in mesoderm migration might be, that the expression of the construct is lower in general. After injection of the pUAST vector into the embryo, the transgene is integrated into the genome randomly. Depending on the genomic environment of the insertion, the expression of the transgene might vary. To avoid this problem, vectors can be used integrate into to the genome at a defined position, so that the expression of transgenes is comparable.

To confirm that phosphorylation of Ser^{825} regulates Rac activation, another phospho-mutation has to be designed. A mutation that changes the Ser^{825} into an Asp (D) would mimic a constitutive phosphorylation. This protein should be able to give a better rescue than the Pbl^{S825A} protein or alternatively result in a dominant phenotype. The exchange activity of both mutants S825A and S825D for Rac and Rho should be tested *in vitro,* to see if there is a decrease or increase of exchange activity. Furthermore we should knock out the whole motif around Ser^{825}, because other Ser are located close to Ser^{825} and might be phosphorylated. Additionally the phosphorylation has to be

shown biochemically with a phospho-specific antibody for Ser^{825} by western blotting. Moreover the localization of phosphorylated Pbl could be investigated in the embryo in vivo. If phosphorylation of Ser^{825} is required for activation of Rac, we would expect a co-localization of Pbl and Rac with the phospho-antibody, but differences in localization detected with the HA-antibody.

The role of the C-terminal tail for the function of Pbl might be to change the substrate preference. Since the absence of The C-terminal tail and the phosphomutation are able to suppress the migration defects. Pbl can bind to both Rho and Rac, but the preference towards binding Rac is enhanced when the Ser^{825} in the C-terminal tail is phosphorylated. Without Phosphorylation and in the absence of the C-terminal tail, Pbl is still able to bind to Rac, but not to such an extent that it is sufficient to induce proper cell migration. The C-terminal tail seems to be required for the substrate preference for Rho as well, because the transgene missing the C-terminal tail is not able to rescue cytokinesis. However it cannot be excluded, that Pbl without the C-terminal tail does not activate Rho at all, because we did not measure the quantity of cytokinesis defects. In summary, a model would be that Pbl has exchange activity for Rac and Rho, the phosphorylation of the C-terminal tail changes or enhances the exchange activity for Rac, whereas dephosphorylation or phosphorylation of a different site in the C-terminal tail changes the exchange activity towards Rho.

Besides the substrate preference, the localization of Pbl at the cell cortex might be important for the function of Pbl in activating Rac during mesoderm migration. Maybe the recruitment to the cell cortex allows the activation of Rac. The C-terminal tail regulates the binding of Pbl to Rac or to other proteins at the cell cortex.

The localization of activated and inactivated Rac in mesoderm cells should be tested. It would be interesting to see if Rac and Pbl co-localize at the cell membrane and if the localization of the activated and inactivated forms is different. Additionally the

localization of Rac targets should be investigated in *pbl* mutant and *pbl* rescued embryos; for instance is the Arp2/3 complex localized to the cell membrane properly in the absence of Pbl.

A model for Pbl function during mesoderm migration

To summarize the previous results a model for Pbl function in mesoderm migration can be drawn (Fig. 5.1): Pbl is recruited to the cell cortex by binding of the PH domain to phospholipids or other membrane binding proteins. The C-terminal tail sustains the cortical binding and the Ser^{825} in the C-terminal tail is phosphorylated by the Htl signalling pathway. This phosphorylation might allow changes in the protein structure resulting in a stronger exchange affinity of the DH domain for Rac. Activation of Rac induces the formation of protrusions at the leading edge of the mesoderm cells by promoting actin filament polymerization and inhibition of actin depolymerization.

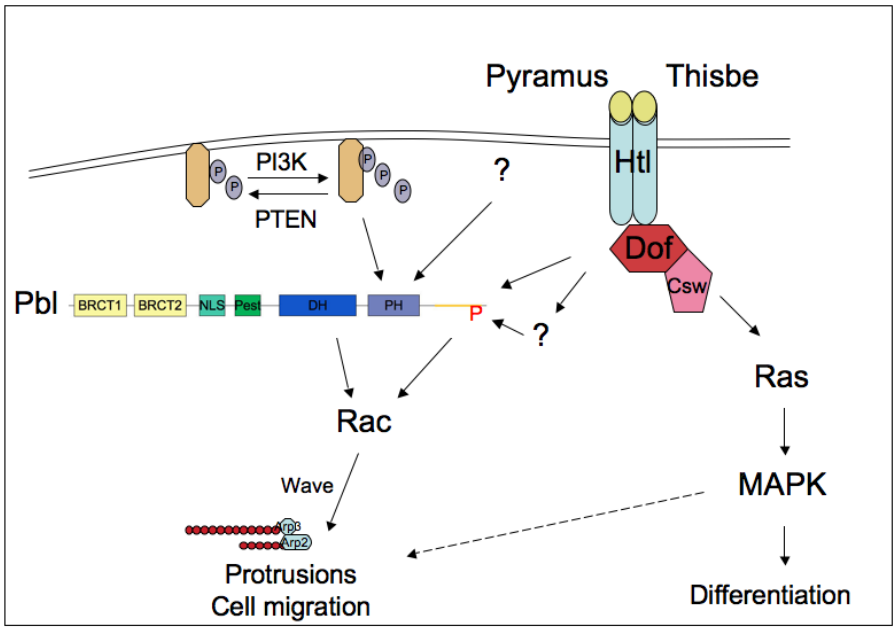


Fig. 5.1 Model of Pbl function in mesoderm cell migration.
After activation of Htl by Ths and Pyr Pbl is recruited to the cell cortex by binding of phospholipids or other proteins to the PH domain. The activation of PI3K is either regulated by Htl activation or through an additional RTK pathway. Then the C-terminal tail binds to the cell cortex and is phosphorylated by Htl or members of the Htl signalling pathway. In parallel Htl activates MAPK, which is required for cell differentiation and might play a role in cell migration. Upon phosphorylation of Pbl it binds and activates Rac via the DH domain. Rac induces the formation of cellular protrusions and probably other processes during cell migration.

For Pbl function in cytokinesis the C-terminal tail has to regulate the switch to a stronger RhoA preference. This can happen by dephosphorylation of the C-terminal tail, or maybe even through phosphorylation of a different phosphorylation site, which was shown for Ect2 *(Niiya et al., 2006).*

5.3 The role of other GTPases in mesoderm migration

Other GTPases are potentially involved in the regulation of mesodermal cell spreading as well. The Rho GTPases RhoA and Cdc42 for example regulate cell migration in other systems *(Nobes and Hall, 1995).*

Additionally the Ras related GTPase Rap1 or Roughened is required for adhesion and spreading of cells in different organisms. Rap1 was shown to regulate activation of Rac during cell spreading, by localizing Rac GEFs to the cell membrane *(Arthur et al., 2004)*. Rap1/Roughened is required for development of the imaginal discs, oogenesis and embryogenesis. Migration of mesoderm and ectoderm cells during dorsal closure are severely perturbed *(Asha et al., 1999).* An important role of Rap1 in FGF signalling was shown during embryogenesis and during the differentiation of photoreceptor cells in the adult eye *(Asha et al., 1999; Franzdottir et al., 2009).* Furthermore an interaction of Rap1 and Pbl was revealed in an eye modifier screen (*Gregory et al., 2007*).

The first function of Rap1 was shown to act opposite to Ras during MAPK activation *(Asha et al., 1999).* However it was shown in other systems, that Rap1 can also act Ras independent. For instance in HeLa cells it was shown that Rap1 directly binds to the GEF Vav2 and recruits it to the plasma membrane, where Vav2 activates Rac and induces cell migration (*Arthur et al., 2004*). Furthermore in migrating rat bladder tumour cells (NBT-II) cells Rap1 acts opposite to Rac1 in cell migration. Rap1 prevents formation of the Paxilin-Crk-DOCK180 complex, which is necessary for Rac

activation (*Vallés et al., 2004*). A possible regulation of Pbl by Rap1 should be investigated further.

Another GTPase required for cell migration is Arf6. Rac1 and Arf6 co-localize to the plasma membrane and recycling endosomes (*Radhakrishna et al., 1999*) and it was shown that Arf6 recruits Rac1 to membrane ruffles (*D'Souza-Schorey and Chavrier, 2006; Myers and Casanova, 2008).* During cell migration membrane traffic by vesicles provides membrane and associated proteins, needed for forward protrusion to the leading edge (*Etienne-Manneville and Hall, 2002; Rodriguez et al., 2003*). Furthermore it is assumed that plasma membrane from the surface of the cell is internalized and transported from the recycling endosomes to the leading edge of the cell, regulated by Arf6 (*de Curtis, 2001*). Since Pbl localization in the cytoplasm and at the membrane always seems to be dotty in the localization studies, Pbl might be localized to vesicles and be recruited in vesicles to the plasma membrane. Immunostaining with an antibody that marks vesicles could be performed to analyze a co-localization of Pbl and vesicles. Moreover Rac, Rho and Cdc42 themselves regulate membrane traffic additionally to their function in actin cytoskeleton organization (*Ridley, 2001*). A possible role for Pbl and Rac in membrane traffic during cell migration should be considered and investigated further.

What happens after Pbl activates Rac?

The mechanisms downstream of Pbl and Rac activation are yet unknown. The modifier screen in this thesis was performed to find genes not only linking Pbl to FGF signalling but additionally genes acting downstream of Pbl. It is necessary to find and investigate other proteins downstream of Pbl and Rac that are required for cell shape changes. Furthermore analysis of the interaction of the other Rho GTPases, RhoA and Cdc42 with Rac during cell migration would be interesting.

It was shown for migrating fibroblasts, that Rac and Cdc42 are both required for the formation of filopodia *(Kraynov et al., 2000; Nobes and Hall., 1995)*; additionally Cdc42 regulates the polarization of the cells and directed migration *(Nobes and Hall, 1995)*. RhoA functions at the rear of the migrating cells in generating a contraction through contractile actin-myosin bundles and focal adhesion complexes, which drives the migration of the cell *(Ridley and Hall, 1994)*. A role for RhoA at the leading edge of migrating cells was described in rat glioma cells. Dia, activated by Rho, is required for the polarity of the migrating cells. Furthermore Dia regulates adhesion turnover via Src and activation of Rac during migration. Therefore Dia is required for catalyzing actin polymerization and regulating microtuble dynamics (MT) in migrating cells *(Yamana et al., 2006)*. Another Rho regulated protein is the Focal adhesion kinase (FAK). FAK is localized to focal contacts and binds to integrins and other scaffolding proteins, which are important for cell migration since they link the cytoskeleton of the cell to the ECM *(Anand-Apte and Zetter, 1997; Playford et al., 2008; Tomar and Schlaepfer, 2009)*. FAK regulates the activation of Rac via phosphorylation of p130Cas *(Playford et al., 2008)*.

The examples of FAK and Dia show that Rho activates Rac via downstream targets during cell migration.

Furthermore RhoA and Rac can even regulate each other's activity positively and negativly. So can RhoA through activation of Rho-kinase induce the activation of Rac *(Salhia et al., 2005; Sinnett-Smith et al., 2001; Tsuji et al., 2002)*; and Rac can induce RhoA activation *(Ridley, 2001)*. One example for Rho and Rac interaction was investigated in *Drosophila* hemocytes in detail *(Williams et al., 2006)*. Hemocytes control the immune response in *Drosophila*. They develop as a cluster of cells from the head mesoderm and migrate and allocate through the whole embryo expressing PVR and following the PDGF/VEGF signals *(Ribeiro et al., 2003)*. Upon receptor activation,

RhoA activation leads to activation of the formin Diaphanous. Dia activates Rac and leads to formation of lamellipodia. Activated Rac then activates the Jun kinase Basket inducing the release of the hemocytes. Basket inhibits RhoGAPp190 resulting in an increase of activated Rho. The hemocytes can now circulate freely until they reach the parasite. RhoA and Basket activate Rho-kinase inducing stress fibres formation and inhibition of Rac activation. Less Rac means less Rho activity resulting in a stable state, which allows the hemocytes to adhere to the parasite *(Williams et al., 2006).*

Besides regulating each other RhoA and Rac can function antagonistically as well. For example during cytokinesis the GAP activity of the centalspindlin complex is required for RhoA activation and Rac inactivation *(Canman et al., 2008).*

All these examples show, that Rho and Rac interact directly and indirectly in many processes. Similar functions for RhoA and Rac during mesoderm cell migration are possible. Since RhoA is required for the invagination of the mesoderm cells and EMT, it is activated during this process and has to be inactivated, or relocated during cell spreading. At the same time Rac has to be activated through Pbl and induce the formation of cellular protrusions. Interplay of Rac at the leading edge and Rho at the reare drives the migration. After the mesodermal cells reach a certain position on top of the ectoderm, they need to stop spreading and form a monolayer. Therefore the formation of protrusions has to arrest by inactivation of Rac. Pbl seems to play a very important role in this interplay and it cannot be excluded that Pbl does not interact with Rho during cell migration at all. Although it was shown that constitutive active Rho does not affect cell migration, whereas active Rac does *(Schumacher et al., 2004),* RhoA still can be involved in the process.

To elucidate the mechanisms that control mesoderm cell migration possible direct and indirect binding partners of Pbl need to be investigated. The screen performed in this thesis revealed potential interactors of Pbl during cell migration.

Characterization of these will hopefully give more insight into the mechanisms up- and down-stream of Pbl and Rac signalling.

5.4 RhoGEF's and cancer

The genetic analysis of cell migration in *Drosophila* can help to understand cell spreading during wound healing and invasion of tumour cells since many of the underlying mechanisms are likely to be conserved in human. Many of the tumour-suppressor genes and proto-oncogenes in *Drosophila*, are important for cell polarity and cell adhesion in numerous tissues during the development of *Drosophila*. Mutations in the human orthologs of the genes that are involved in the loss of cell polarity and epithelial cell adhesion are involved in the progression and metastasis of various tumours *(Wodarz and Nähtke, 2007).*

In many tumour cells RhoGEFs are overexpressed, resulting in EMT and reorganization of the actin cytoskeleton and leading to cell invasion and metastasis. Interestingly, Ect2 is overexpressed in brain, pancreatic, lung, bladder, oesophageal and ovarian tumour cells *(Saito et al., 2004; Sano et al., 2006; Salhia et al., 2008; Zhang et al., 2008; Hirata et al., 2009).* The depletion of Ect2 in glioma cells leads to a decrease in proliferation and invasion of these cells *(Sano et al., 2006; Salhia et al., 2008).* Furthermore multi-nucleation was found in these glioma cells *(Salhia et a., 2008),* indicating that loss of Ect2 interferes with RhoA and Rac function in cancer cells. A significant decrease of Rac activity was shown in Ect2 KD lung cancer cells *(Justilien and Fields, 2009).* Ect2 associates with the oncogenic PKC/Par6 complex and activates Rac. PKC functions downstream of Ras and upstream of Rac and the tumour cells lose their polarity, which is similar in *Drosophila* after depletion of DaPKC *(Murray et al., 2004).* The involvement of Ect2 in all these different tumour types shows, that it is important to investigate the regulation of Ect2 and find ways in form of therapeutical methods to inhibit its function or better to restore it in cancer patients. Due to the strong

homology between Pbl and Ect2 the investigation of Pbl function is important as well and can lead to a better understanding of Ect2 regulation and function.

Furthermore the importance of *Drosophila* as a model organism was shown not only for cancer but also for several human diseases like neuromuscular diseases *(Lloyd and Taylor, 2010)*, asthma *(Roeder et al., 2009)*, cardiac diseases *(Bier and Bodmer, 2004)* and many more. These examples illustrate the potential of investigating disease-related genes like Pbl in *Drosophila*.

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Appendix

Bloomington number	Deletion name	Cytological region	Genetic interaction in the eye	Mesoderm defects
#90	Df(2L)C144	22F4-23A1; 23C2-4	suppression	sporadic migration defects
#7494	Df(2L)Exel6008	22F4;23A3	?	no defects
#7744	Df(2L)Exel6277	23A2;23B1	supression	no defects
#1567	Df(2L)JS17	23C1-2; 23E1-2	enhancement	11 eve cell cluster, sporadic migration defects
#7784	Df(2L)Exel7014	23C4;23C5	enhancement	11 eve cell cluster, sporadic migration defects
#8038	Df(2L)ED206	23B8;23C5	no interaction	/
#6875	Df(2L)BSC28	23C5-D1; 23E2	no interaction	/
#693	Df(2L)sc19-8	24C8-24D5	suppression	no eve cell cluster, migration defects
#744	Df(2L)M24F-B	24E1-2; 24F6-7	suppression	no defects
#702	Df(2L)ed-dp	24C3-5; 25A2-3	suppression	no eve cell cluster, migration defects
#7495	Df(2L)Exel6009	24C3;24C8	no interaction ?	no eve cell cluster, weak migration defects
#7789	Df(2L)Exel7018	24A1;24C2	enhancement/ suppression ??	no defects
#7790	Df(2L)Exel8010	24C8;24D4	/	no defects
#7793	Df(2L)Exel8012	25B1;25B5	no interaction	/
#7796	Df(2L)Exel8013	25B5;25B10	no interaction ?	/
#3812	Df(2L)sc19-3	24E3;25A7	enhancement	no defects
#652	Df(2L)sc19-6	24F1-2; 25C3-5	enhancement	no defects
#7496	Df(2L)Exel6010	25A7;25B1	enhancement	no defects
#7497	Df(2L)Exel6011	25C8;25D5	/	no defects
#9600	Df(2L)BSC165	24D4;24D8	no interaction	/
#9694	Df(2L)BSC217	24D8;24F1	no interaction ?	/

#9695	Df(2L)BSC218	24D8;24E1	no interaction	/
#9270	Df(2L)ED250	24F4;25A7	enhancement	/
#490	Df(2L)E110	25F3-26D11	suppression	sporadic migration defects
#8902	Df(2L)ED320	25F2;26B2	suppression	sporadic migration defects
#9272	Df(2L)ED347	25F5;26B5	no interaction	/
#7798	Df(2L)Exel8016	25E6;25F2	/	no defects
#9343	Df(2L)ED334	25F2; 26B2	/	no defects
#8040	Df(2L)ED284	25F2; 26A3	no interaction	/
#5420	Df(2L)Dwee1-W05	27C2-3; 27C4-5	suppression	sporadic migration defects
#8847	Df(2L)BSC108	27C1;27C6	suppression	no defects
#2414	Df(2L)spd[j2]	27C1-2;28A	no interaction	/
#8940	Df(2L)ED6569	27A1;27C4	suppression ?	/
#463	Df(2L)BSC255	35D3;35D4	suppression	no defects
#2583	Df(2L)cact-255rv64	35F-36D	enhancement	10 eve cell cluster, sporadic migration defects
#1491	Df(2L)r10	35D1; 36A6--7	no interaction	/
#420	Df(2L)TW137	36C2-4; 37B9-C1	no interaction	/
#7833	Df(2L)Exel7066	36A1;36A12	no interaction	/
#7522	Df(2L)Exel6039	36A10;36B3	enhancement	sporadic migration defects
#7835	Df(2L)Exel8036	36B1;36C9	no interaction	/
#3518	Df(2R)Jp1	51D3-8; 52F5-9	suppression	no defects
#3520	Df(2R)Jp8	52E1-4; 53B5-C1	suppression	no defects
#8915	Df(2R)ED2457	52D11;52E7	suppression	/
#3521	Df(2R)Jp6	52E3-5;52F	suppression	/
#6455	Df(2R)BSC11	50E6-F1; 51E2-4	suppression weak	no defects
#1150	Df(2R)kmSA3	51B5-11; 51D7-E2	suppression	/
#7749	Df(2R)Exel6284	51B1; 51C2	no interaction	/
#6380	Df(2R)kmSA4	51C3; 51D6	no interaction	/
#7445	Df(2R)BSC49	53D9-E1; 54B5-10	enhancement	no defects
#7887	Df(2R)Exel7145	53D4;53D12	enhancement	/
#9278	Df(2R)ED2747	53D11;53F8	enhancement	/

#3650	Df(3L)M21	62F;63D	suppression	/
#3649	Df(3L)HR119	63C2;63F7	suppression	11 eve cell cluster, more eve cells (5-7), sporadic migration defects
#3647		63A1;63D1	suppression	/
#7929	Df(3L)Exel8104	65F7;66A4	suppression	no defects
#1541	Df(3L)ZP1	66A17-20; 66C1-5	suppression	no defects
#7745	Df(3L)Exel6279	66A17; 66B5	enhancement	missing eve cell cluster, migration defects
#8065	Df(3L)ED4408	66A22; 66C5	suppression	/
#7591	Df(3L)Exel6112	66B5; 66C8	suppression	no defects (sporadic defects)
#4500	Df(3L)Scf-R6	66E1-6	enhancement	sporadic migration defects
#7079	Df(3L)BSC35	66F1-2; 67B2-3	suppression	no defects
#3024	Df(3L)h-i22	66D10-11; 66E1-2	no interaction	/
#2611	Df(3L)vin5	68A2-3; 69A1-3	suppression	no defects
#2612	Df(3L)vin7	68C8-11; 69B4-5	suppression	no defects
#8068	Df(3L)ED4470	68A6;68E1	suppression	/
#2993	Df(3L)st-f13	72C1-D1; 73A3-4	suppression	more eve cells, migration defects
#3640	Df(3L)brm11	71F1-4; 72D1-10	no interaction	/
#2998	Df(3L)81k19	73A3;74F	no interaction	/
#6411	Df(3L)BSC8	74D3-75A1; 75B2-5	suppression	no defects
#8099	Df(3L)ED4685	73D5;74E2	suppression	/
#8100	Df(3L)ED4710	74D1;75B11	suppression	/
#3617	Df(3L)kto2	76B1-2;76D5	suppression	sporadic migration defects
#5087	Df(3L)BSC2	76C;76F2-3	suppression	no defects
#8087	Df(3L)ED229	76A1;76E1	suppression	/
#5126	Df(3L)XS533	76B4;77B	suppression	11 eve cell cluster, sporadic

#5126	Df(3L)XS533	76B4;77B	suppression	11 eve cell cluster, sporadic migration defects
#1842	Df(3R)Antp17	84D3-84D9	suppression	missing eve cell cluster, migration defects
#1941	Df(3R)Antp2	84B2;84D3	enhancement	no defects
#9201	Df(3R)ED5221	84C4;84E11	no interaction	/
#9076	Df(3R)ED5223	84D9;84E11	suppression	no defects
#2393	Df(3R)WIN11	83E1-2 ; 84A5	/	strong invagination defects
#7625	Df(3R)Exel6146	84C8;84D9	enhancement	/
#8685	Df(3R)ED7665	84B4;84E11	/	no defects
#1968	Df(3R)p712	84D4-85B6	enhancement	sporadic missing eve cell cluster
#7627	Df(3R)Exel6148	84F12;85A2	enhancement	no defects
#9338	Df(3R)ED5296	84F6;85C3	no interaction	/
#1847	Df(3R)dsx2D	84D11;84F16	no interaction	/
#4431	Df(3R)DG2	89E1-F4 ; 91B1-B2	suppression	migration defects
#7657	Df(3R)Exel6178	90F4;91A5	suppression	no defects
#9208	Df(3R)ED5815	90F4;91B8	suppression	no defects
#669	Df(3R)Dr-rv1	99A1-2 ; 99B6-11	enhancement	no defects

Table 1. Deletions on the second and third chromosome tested for interaction with *Pbl* $^{DH\text{-}PH}$ in the eye modifier screen.

Fly stocks carrying deletions show either suppression (green), enhancement (red) of the rough eye phenotype or no interaction (white).

Cytological region	Name gene	Name stock	interaction
23C5-D1	CG17219	PBacPBCG17219/Cyo	no
	GABPI	PBacPBCG17257^{c06930}	no
	CG17259	P{SUPorP}CG17259^{KG03126}/CyO; ry^{506}	no
	CG17221	PBac{PB}CG17221^{c00569}/CyO	no
	v(2)k05816	P{EP}v(2)k05816^{EP695}/CyO	no
	CG3523	P{SUPor-P}CG3523^{KG03696}/CyO, P{sevRas1.V12}FK1	no
	toc	toc^{A1-1} cn^{1} bw^{1}/CyO	enhancement
		toc^{A1-7} cn^{1} bw^{1}/CyO	enhancement
	PpD6	P{SUPor-P}toc^{KG08989} PpD6^{KG08989}	no
36A12-B1	VhaSFD	P{EPgy2}VhaSFD^{EY04644}/CyO	no
	CG17996	P{EPgy2}CG17996^{EY20718}	no
	CG17331(Lsm7)	PBac{PB}CG17331^{c05852}	no
	glu	glu$^{88-37}$/CyO	no
	ChlD3	P(EP)EY01205	no
	CG17912	P{EP}CG17912^{EP819}/CyO	no
51C2-3	Rpn6	Rpn6^{2F}/CyO, P{ActGFP}JMR1	no
	CG10151	P{EPgy2}CG10151^{EY03966}	weak enhancement
	CG12857	P{XP}CG12857^{d01332}	no
	CG12854	P{EPgy2}Spred^{EY04308}	?
	CR30478 (CG42254)	PBac{RB} CR30478^{e00165}	?
	BEAF-32	P{SUPor-P}BEAF-32^{KG06904}	no
53D11-14	CG5859	P{EP}CG5859^{EP2090}	no
	Dek	P{EPgy2}Dek^{EY07989}	weak enhancement
	Ef1β	P{EPgy2}Ef1β^{EY05513}	no
	CG6426	P{lacW}CG6426^{k04810}	no
76D2-3	Rab8	PBac{5HPw+}Rab8^{B229}/TM3, Sb^{1} Ser^{1}	weak suppression/no
	Mi-2	P{EPgy2}Mi-2^{EY13252}	no
	Su(Tpl)	Su(Tpl)^{10} kni^{ri-1} Ki^{1} p^{p}/TM3, Ser^{1}	no
99A1-A6	CG31445	P{SUPor-P}CG31445^{KG01444a} P{SUPor-P}stg^{KG01444b}	weak enhancement
	CR31044	P{Mae-UAS.6.11}Ef1γ^{UY752} CR31044^{UY752}	enhancement
	CG14507	PBac{PB}CG14507^{c00851}	weak enhancement
	CG15817	PBac{PB}CG15817^{c05664}	weak enhancement
	CG11951	PBac{WH}CG11951^{f00339}	no

Table 2. Genes tested for interaction with *Pbl*$^{DH-PH}$ in the eye modifier screen.
The table shows fly stock, which carry either mutations or P-element insertions in genes, located to modifier regions found in the screen.

EMS mutants	Chromosome arm	Fertile	Twi staining defects	Eve staining defects
Su (3) 18	3L	+	sporadic migration defects	-
	3R	-	-	-
Su (3) 29	3L	+	-	less eve clusters
	3R	-	-	-
Su (3) 21-10-3	3L	-	-	-
	3R	+	-	-
Su (3) 21-10-4	3L	-	-	-
	3R	few embryos	-	-
Su (3) 21-10-30	3L	-	-	-
	3R	-	-	-
Su (3) 26-10-3	3L	+	-	-
	3R	+	-	-
Su (3) 26-10-27	3L	-	-	-
	3R	+	-	-
Su (3) 26-10-31	3L	+	-	less eve clusters
	3R	-	-	-
Su (3) 28-10-3	3L	-	-	-
	3R	few embryos	-	-
Su (3) 28-10-8	3L	+	-	-
	3R	few embryos	-	-
Su (3) 29-10-3	3L	-	-	-
	3R	-	-	-
Su (3) 29-10-7	3L	+	migration defects	less Eve clusters
	3R	+	defects	-
Su (3) 31-10-1	3L	+	-	-
	3R	few embryos	weak defects	-
Su (3) 3-11-4	3L	-	-	-
	3R	-	-	-
Su (3) 3-11-53	3L	+	migration defects	-
	3R	+	polarity, invagination defects	
Su (3) 10-11-1	3L	+	-	-
	3R	-		
Su (3) 10-11-14	3L	+	-	-
	3R	few embryos	-	-
Su (3) 17-12-2	3L	-	-	-
	3R	few embryos	-	-
Su (3) 25-12-4	3L	-	-	-
	3R	+	-	-

Tab. 3. Germline clones of EMS suppressor mutants identified in the EMS screen.
The table shows embryos of females, which are recombinant for both arms of the chromosomes. The embryos are lacking maternal gene product. Eight mutations cause defects during embryonic development.

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